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Docket No.: 02427/100G772-US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Erich HOFFMANN

Serial No.: 09/844,517 Group Art Unit: 1648

Confirmation No.: 9063 Examiner: Myron G. Hill

Filed: April 27, 2001

For: DNA TRANSFECTION SYSTEM FOR THE GENERATION OF
INFECTIOUS INFLUENZA VIRUS

DECLARATION OF RICHARD J. WEBBY UNDER 37 CFR § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, RICHARD J. WEBBY, do hereby declare and state the following:

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Serial No.: 09/844,517
Filed: 04/27/2001
Group Art Unit: 1648

1. I am an Assistant Member at the Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee.

2. I was awarded a Ph.D. degree in Virology in 1998 from the University of Otago, New Zealand. Upon completion of my doctorate, I worked as a Research Assistant at the Department of Microbiology at the University of Otago, New Zealand. In 1999, I joined the Division of Virology, Department of Infectious Diseases at St. Jude Children's Research Hospital, Memphis, Tennessee as a Postdoctoral Fellow and in 2003 became an Assistant Member. During my entire career I have been working in the field of virology. Since 1999, my research has been focused on influenza virus biology and production of recombinant vaccines. A copy of my Curriculum Vitae is annexed as Exhibit 1.

3. I make this Declaration in support of the application identified above ("the '517 application"). I am not an inventor of the '517 application and I do not have any financial interest in this application.

4. I have reviewed the entire disclosure of the '517 application, including the original claims and the drawings, as well as the prosecution history and claims, as amended in the accompanying amendment. I have also specifically reviewed the Final Office Action dated May 5, 2004, which was issued in connection with this application.

5. I understand that in the Final Office Action dated May 5, 2004, the United States Patent and Trademark Office has rejected claims 15-17, 19-30, 32, 39, and 44 of the '517 application under 35 U.S.C. § 103(a) on the grounds that the claims are allegedly obvious over Hoffmann dissertation (1997) and Neumann *et al.* (Proc. Natl. Acad. Sci., 1999, 96: 9345-50)

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and has rejected claim 45 as being allegedly obvious over Hoffmann and Neumann *et al.* and further in view of Pleshka *et al.* (J. Virol., 1996, 70: 4188-92).

6. In the Office Action, the Examiner has stated that, knowing that Neumann *et al.* (i) generated infectious influenza virus using a plasmid-based system and (ii) disclosed the benefit of adding more protein expressing plasmids to the transfection, and also knowing that multi-plasmid transfections are complex, one of ordinary skill in the art at the time of the invention would be motivated to come up with the present invention by using the plasmid described in the Hoffmann dissertation to reduce the number of plasmids for transfection and to save time in cloning. The Examiner appears to believe that there would be an expectation of success, because the promoter elements used by Hoffmann are the same as used by Neumann *et al.*

7. Based on the materials I reviewed and my experience in the field of influenza virology and recombinant vaccine generation, it is my opinion that the Hoffmann dissertation and Neumann *et al.* article do not suggest or provide any expectation of success for the creation of the dual pol I-pol II promoter plasmid system for the generation of infectious influenza viruses from cloned viral segments as disclosed in the '517 application and recited in the present claims. My detailed explanation is provided below.

8. The '517 application discloses and claims a novel reverse genetic system for generation of infectious influenza viruses. This system comprises a set of plasmids (each plasmid being a dual promoter pol I-pol II plasmid comprising one influenza viral genomic segment) which, when co-introduced into a host cell in the absence of any helper virus or additional viral protein-expressing plasmids, produce an infectious influenza virus. In other

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words, there are three distinguishing features of the plasmid composition of the present invention: (1) the presence of both pol I and pol II promoter on the same plasmid encoding a viral gene segment; (2) the total number of plasmids corresponds to the total number of gene segments from the source virus (*e.g.*, 8 plasmids for an 8-segmented influenza A virus), and (3) the ability to reconstitute an infectious virus (upon plasmid transfection into a host cell) in the absence of any helper virus or additional viral protein-expressing plasmids.

9. The Examiner states that the reverse genetic system of the '517 application is obvious in light of the inventor's (Hoffmann) 1997 Ph.D. dissertation, which describes dual pol I-pol II promoter plasmids. This reference does not support such a conclusion. The Hoffmann dissertation merely describes a model pol I-pol II plasmid carrying a non-viral reporter gene (*e.g.*, CAT or GFP) which replicates in the presence of viral polymerase proteins supplied by a helper virus. The Hoffmann dissertation provides no suggestion to make a pol I-pol II plasmid encoding a viral gene segment. It also does not provide any basis for expecting to generate an infectious influenza virus using 8 pol I-pol II plasmids without a helper virus. The pol I-pol II reporter gene construct described in the Hoffmann thesis is an interesting curiosity. However, even if one were to extrapolate from this construct to a construct with a viral gene instead of a reporter gene, there remain vast uncertainties as to whether the expression of vRNA and mRNA from that construct would yield functional RNA molecules. To then leap to the 8-plasmid dual pol I-pol II promoter system of the present invention goes orders of magnitude beyond what the Hoffmann dissertation actually teaches or suggests. Each of the 8 dual promoter plasmid constructs introduces a degree of uncertainty, further compounded when one omits a helper virus.

10. The Neumann *et al.* article cited by the Examiner describes a very different reverse genetic system from the one disclosed in the '517 application. Specifically, the reverse genetic system of Neumann *et al.* achieves generation of an infectious influenza virus by using a totally different set of plasmids (as compared to the plasmid system of the '517 application): (1) pol I-only plasmids directing the synthesis of vRNA/cRNA from a pol I promoter and (2) pol II-only plasmids directing the synthesis of mRNA encoding viral proteins from a pol II promoter (*i.e.*, at least plasmids encoding viral polymerase proteins PB1, PB2, PA, and NP). It follows that, while the dual pol I-pol II promoter plasmid system of the '517 application uses the number of plasmids equal to the number of viral genomic fragments (*e.g.*, 8 plasmids for 8-segmented influenza A virus), the reverse genetic system Neumann *et al.* uses the total number of plasmids which exceeds the total number of viral genomic segments (*i.e.*, at least 12 plasmids for an 8-segmented influenza A virus). In fact, in its most efficient (high yield) versions, the system of Neumann *et al.* uses extra protein expression (pol II) plasmids encoding HA, NA, M1, M2, and NS2 viral proteins (making the total number of transfected plasmids 17 instead of 12; see, p. 9347 [~~¶ bridging left and right col.~~] and Table 1 at p. 9348 of the Neumann *et al.* article).

11. The separation of pol I and pol II promoters on different plasmids in the reverse genetic system of Neumann *et al.* was believed to be one of the main reasons for successful generation of an infectious virus. Such separation allows control and regulation of viral genome replication and protein expression and in this way allows one to recapitulate more closely the life cycle of the influenza virus, which is characterized by strict spatial and temporal regulation of transcription and replication.

12. The temporal and spatial regulation of the influenza virus replication and transcription can be briefly summarized as follows (see, *e.g.*, Steinhauer and Skehel, Ann. Rev. Genet., 2002, 36: 305-332 and Whittaker, Expert Reviews in Molecular Medicine, 8 February 2001, attached as Exhibits 2 and 3, respectively). Influenza transcription and replication occurs in the host cell nucleus. Four proteins, PB1 (viral transcriptase), PB2 (viral endonuclease), PA, and NP (nucleoprotein), are essential for replication and transcription of the viral genome. Genome segments are transcribed by the three polymerase polypeptides associated with each genome segment: PB2 attaches to the m7G cap of host mRNAs, this structure is cleaved from the mRNA by PB1, remaining attached to PB2. The cap serves as a primer for RNA synthesis and 11-15 nucleotides are added by PB1, after which PB2 dissociates from the growing strand. PB1 and PA then complete the synthesis of the (+) sense strand. Two classes of (+) sense RNA are made in infected cells: (1) incomplete, 3' polyadenylated transcripts, which are exported to the cytoplasm and serve as mRNAs and (2) cRNA, complete, non-polyadenylated (+) sense copies of the (-) sense vRNA, which serve as template for the synthesis of progeny (-) sense vRNAs. Double stranded (+/-) replicative intermediate structures can be isolated from the nucleus of infected cells. Most of the proteins made (*e.g.*, hemagglutinin (HA), neuraminidase (NA), matrix proteins (M1 and M2), and NS proteins (NS1 and NS2)) remain in the cytoplasm or become associated with the cell membrane. However, the NP protein migrates back into the nucleus, where it associates with newly-synthesized vRNA to form new nucleocapsids. These migrate back out into the cytoplasm and towards the cell membrane. The level of free NP is thought to control whether mRNA or cRNA is produced, *i.e.*, later in infection when there is lots of NP, mRNA synthesis stops but cRNA synthesis continues.

13. The Neumann *et al.* system allows one to regulate viral segment replication and protein expression by using pol I-only replication plasmids and pol II-only protein expression plasmids in a manner that more closely resembles the natural influenza life cycle. Efficient generation of the infectious virus in the Neumann *et al.* system correlates with the ability to provide specified amounts of each viral protein by transfecting different amounts of each of the protein expression (pol II) plasmids (see, e.g., p. 9347 left col., ¶¶ 3-4 of the Neumann *et al.* article). With these facts in mind, there would be a disincentive to use a reverse genetic system that lacked such controls.

14. In contrast to the Neumann *et al.* system, in the dual pol I-pol II promoter system of the present invention, equal amounts of each plasmid can be used, and vRNA/cRNA and mRNA syntheses occur from the same plasmid and cannot be separately controlled. In view of researchers in the field of influenza virology at the time when Dr. Hoffmann came up with the dual pol I-pol II system, this created significant spatial and temporal hurdles for regulation of transcription and replication, which are not present either in the Neumann *et al.* system or in nature. For example, spatial and temporal hurdles stem from the fact that pol I-mediated transcription (vRNA/cRNA synthesis) takes place in the nucleolus, while pol II-mediated transcription (mRNA synthesis) takes place in a different site in the nucleus. Since it was not known whether mRNA and vRNA/cRNA syntheses occur on pol I-pol II plasmids at the same time, the ability of these plasmids to produce both mRNA and vRNA/cRNA appeared uncertain. Also, it appeared uncertain that pol I- and pol II-directed transcription (involving assembly of huge molecular complexes and conformational changes of the template) can occur successfully on the same artificial construct in the absence of natural viral regulatory mechanisms. Indeed, mRNA transcripts derived from pol I-pol II plasmids contain pol I promoter and terminator

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sequences in their 5' and 3' non-coding regions. These sequences (not present in the Neumann *et al.* system) are sites where pol I transcription and termination factors bind. One could therefore envision that binding of these factors to pol I promoter and terminator regions in the pol I-pol II plasmids may result in reduction in pol II-mediated transcription. The interaction of pol I promoter and terminator sequences with influenza sequences in mRNA derived from pol I-pol II plasmids was also unpredictable and could possibly affect mRNA stability and translation efficiency leading to a lower abundance of some or all mRNAs. This is precisely why Dr. Hoffmann's colleagues (among them several prominent experts in the field) were skeptical that the dual pol I-pol II promoter system of the present invention would ever generate an infectious virus. The success of the present invention came as a great surprise.

15. Once Dr. Hoffmann established that the pol I-pol II plasmid-based system would work, its advantages became apparent: it is much simpler and more reproducible than the system of Neumann *et al.*, because it significantly decreases the number of plasmids for transfection and eliminates the need to adjust the relative amount of each plasmid. Also, the Neumann *et al.* system as originally developed and published was capable of efficiently producing infectious virus only in 293T cells. 293T cells are transformed cells and therefore cannot be approved by WHO for vaccine seed production. In contrast, the 8-plasmid dual pol I-pol II promoter system of the present invention permitted efficient generation of infectious viral particles in WHO-approved African green monkey kidney (Vero) cells.

16. I and co-workers have successfully used the 8-plasmid dual pol I-pol II promoter system of the present invention for the production of a number of recombinant vaccines. For example, we have recently generated a vaccine to the deadly avian H5N1 influenza virus

A/Hong Kong/213/03, which caused human infections and lead to a WHO pandemic alert on February 19, 2003 (see Webby *et al.*, Lancet, 2004, 363: 1099-103; attached as Exhibit 4). Specifically, the use of the plasmid system of the present invention allowed us to remove the polybasic amino acids from the HA cleavage site, which are associated with high virulence of the H5N1 virus, and produce a reference vaccine virus on an A/Puerto Rico/8/34 (PR8) backbone in WHO-approved Vero cells. The modified virus proved to be non-pathogenic in chickens and ferrets and was shown to be stable after multiple passages in embryonated chicken eggs (where the final vaccine is currently produced). The successful generation of the H5N1 vaccine provides a clear proof that the dual pol I-pol II promoter system of the present invention is a significant scientific breakthrough, which has the potential to revolutionize the way we prepare and manufacture pandemic and interpandemic influenza vaccines. We have also used the dual pol I-pol II promoter system of the present invention to create a vaccine reference virus to A/Vietnam/1203/04, a virus representative of those circulating in Southeast Asia. In addition, we have shown in proof-of-principle experiments that H1N1, H3N2, H6N1, and H9N2 influenza viral strains can be rescued in Vero cells using the 8-plasmid dual pol I-pol II promoter system of the present invention (see Ozaki *et al.*, J. Virol., 2004, 78: 1851-1857; attached as Exhibit 5).

17. For the reasons provided above, it is my opinion that the present invention is not obvious over the Hoffmann dissertation and Neumann *et al.* article. The Hoffmann thesis simply lacks enough guidance to employ a pol I-pol II construct containing a viral segment, much less a set of them, to generate an infectious virus in the absence of a helper virus. Certainly nothing in Neumann *et al.* reference would have led one to use a pol I-pol II plasmid. On the contrary, the Neumann *et al.* system appeared to work better with more, not fewer, plasmids, which provide greater control. In any case, there was no way to know whether the pol I-pol II plasmid approach

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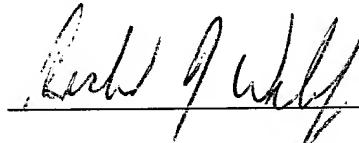
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of the present invention would work, until Dr. Hoffmann showed that it worked. Remarkably, the plasmid system of the present invention is not only simpler, but it is also efficient, another unexpected benefit, especially in light of Neumann *et al.* demonstrating greater efficiency with more plasmids. Accordingly, it is my opinion that, even if artificially combined, the references cited by the Examiner do not provide any suggestion or motivation to generate the presently claimed set of pol I-pol II plasmids, much less provide any expectation of success of using these plasmids for the generation of infectious influenza viruses entirely from cloned viral segments.

18. I hereby declare that all statements made herein of my own knowledge are true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

10/20/2004

Date:



Richard J. Webby, Ph.D.

CURRICULUM VITAE

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Honors

Proudfoot Award in Experimental Science – undergraduate award for excellence in experimental science, 1991
 Otago University Postgraduate Scholarship awarded for PhD study, 1992

B. Selected peer-reviewed publications (in chronological order).

Webby RJ, Kalmakoff J. Sequence comparisons of the major capsid protein gene from 18 diverse iridoviruses. *Arch Virol* 143(10):1949-1966, 1998.

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GENETICS OF INFLUENZA VIRUSES

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Key Words mutants, antigenicity, reassortment, recombination, pathogenicity

■ Abstract Influenza A viruses contain genomes composed of eight separate segments of negative-sense RNA. Circulating human strains are notorious for their tendency to accumulate mutations from one year to the next and cause recurrent epidemics. However, the segmented nature of the genome also allows for the exchange of entire genes between different viral strains. The ability to manipulate influenza gene segments in various combinations in the laboratory has contributed to its being one of the best characterized viruses, and studies on influenza have provided key contributions toward the understanding of various aspects of virology in general. However, the genetic plasticity of influenza viruses also has serious potential implications regarding vaccine design, pathogenicity, and the capacity for novel viruses to emerge from natural reservoirs and cause global pandemics.

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INTRODUCTION

Influenza A viruses are lipid-enveloped viruses containing a genome composed of eight strands of negative-sense RNA that encode ten viral proteins. These gene segments are encapsidated in a virally encoded nucleoprotein (NP), and the ribonucleoprotein (RNP) structures are associated with the three subunits of the viral polymerase (PB1, PB2, and PA). Virus particle formation occurs at the surface membrane of infected cells, where budding occurs from regions of the membrane at which the viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA), have accumulated. The viral matrix protein (M1) is the most abundant component of the virion and is thought to play a pivotal role in the process of assembly and budding. Details on the mechanism of assembly are still forthcoming, but it is often assumed that M1 interacts with the RNPs and the cytoplasmic domains of HA, NA, and possibly the third integral membrane protein M2. The two other viral proteins, NS1 and NS2, were initially designated as nonstructural proteins, but there is now evidence for the presence of NS2 in virions.

Depending on the virus strain and passage history, influenza A virions can exhibit a variety of shapes and sizes, ranging from fairly spherical particles of approximately 100 nm in diameter to elongated filamentous forms of the virus (69, 71). Initial human isolates tend to be largely of the filamentous type (26), but upon continuous propagation in the laboratory, viruses with more spherical morphology can be selected (25). Regardless of the virion morphology, the most prominent feature of the virus envelope is the layer of tightly packed HA and NA glycoproteins that project from the viral membrane.

STRUCTURE OF THE INFLUENZA GENOME

Segmented Nature of the Genome and Reassortment

It was not until the 1960s that tissue culture systems for growth and plaque purification of influenza were well established. As a consequence, many of the pioneering studies on influenza were dependent on characteristics such as their ability to grow in allantoic membrane cells of embryonated chicken eggs and their capacity to agglutinate erythrocytes (hemagglutination), which made it possible to approximate virus yields. The early studies of influenza genetics also relied heavily on the availability of genetic markers such as neurovirulence, antigenicity, hemagglutination properties, and virus morphology. These markers were not ideal since the basis of the phenotype was ill defined and often polygenic in nature. Despite this, it was clear that during coinfection with viruses of differing phenotypes, recombination

of such markers occurred at frequencies much higher than could be explained by classical microbial genetics. This was first observed by Burnet & Lind in 1949 (19), who reported that antigenicity and neurotropism phenotypes could be segregated following virus infection of mouse brains. The high frequencies of recombination that they noted in this and similar studies led them to speculate that "influenza particles are composite, and on entry into the host cell break down into subunits which replicate independently, giving rise to a pool of virus material from which infectious units can be reconstructed" (20).

In 1951 Henle & Liu (60) showed that UV-inactivated influenza viruses could regain infectivity if cells were infected at a multiplicity greater than one, a phenomenon referred to as "multiplicity reactivation." It was subsequently shown that UV-inactivated virus preparations reactivated by coinfection with infectious viruses can display inheritable characteristics derived from each parent, indicating that the inactivated virus had become infectious by acquiring additional genetic material (5). Single cycle kinetic experiments on multiplicity reactivation led Barry (7) to speculate that the influenza genome might be composed of approximately six independent units as targets of UV inactivation. The numerous observations of high-frequency recombination by influenza viruses compared with other RNA viruses such as poliovirus and Newcastle disease virus, and studies on influenza virus defective interfering (DI) particles that were genetically incomplete, led Hirst to formally propose that the influenza genome was composed of RNA fragments (63). Furthermore, he postulated that for influenza virus there might be "two kinds of recombination," one of which "occurs at high rate and involves the exchange of large pieces." This type of recombination, in which individual gene segments or combinations of segments are exchanged during mixed infections, is referred to as reassortment, and the viruses that result from such genetic exchanges are termed reassortant viruses.

Description of the Gene Segments

With the advent of improved electrophoretic techniques for separating nucleic acids and proteins, it was demonstrated that the influenza A genome consists of eight separate RNA segments (8, 103, 123, 132). In this section we identify the gene segments and give a brief description of the gene products. Details on the RNA structure of the gene segments, the functions of the viral proteins, and an extensive list of relevant references have recently been reviewed (87).

Using the A/Puerto Rico/8/34 virus (PR8) as an example, the influenza A viral gene segments range in size from 890 to 2341 nucleotides and contain from 20 to 45 noncoding nucleotides at the 3' end and 23 to 61 nucleotides at the 5' end, depending on the segment. With the exception of position 4 at the 3' end of viral gene segments, which displays U/C heterogeneity, the 12 nucleotides at the 3' end and the 13 nucleotides at the 5' end are completely conserved for all segments in all strains of influenza A virus. These terminal RNA regions are partially complementary and viral promoter activity has been mapped to these domains, but with the exception

of the polyadenylation signal, the functional significance of most of the noncoding sequences beyond the conserved domain remains unresolved. There is common speculation that these sequences are involved in binding of NP and/or polymerase complexes, or that they may contribute to packaging signals.

The three largest gene segments encode the subunits of the viral polymerase, PB2, PB1, and PA, which are so named because of their basic (PB2, PB1) or acidic (PA) properties on isoelectric focusing gels. These are responsible for transcribing messenger RNAs (mRNAs), for synthesizing positive-sense antigenomic template RNAs (cRNAs), and for transcribing the cRNAs into the gene segments (vRNAs) that are incorporated into progeny viruses. Segment 4 encodes the hemagglutinin glycoprotein (HA), which is responsible for binding virus to sialic acid-containing cell-surface receptors and for membrane fusion during virus entry into host cells. It is also the principal target for neutralizing antibodies. The nucleoprotein (NP) is the product of the fifth gene segment. This is the protein that encapsidates cRNAs and vRNAs, which is necessary for them to be recognized as templates for the viral polymerase. Segment 6 encodes the neuraminidase (NA), which cleaves sialic acid from virus and host cell glycoconjugates at the end of the virus life cycle to allow mature virions to be released. Segment 7 generates two gene products, the matrix protein, M1, and the M2 protein. M1 mRNA is a polycistronic transcript, and its product has a structural role in the virion and it is thought to play a fundamental role in virus assembly. The M2 is a small transmembrane protein derived from spliced mRNA. It has proton channel activity that aids in virus disassembly during the initial stages of infection. The eighth gene segment also encodes two proteins due to alternative splicing. These proteins were originally referred to as NS1 and NS2 because they were thought to be nonstructural, but NS2 has since been shown to be a component of virions. The NS1 has numerous functions. It is a regulator of both mRNA splicing and translation, and it also plays a critical role in the modulation of interferon responses to viral infection. The NS2 functions to mediate the export of newly synthesized RNPs from the nucleus and as such, it is also referred to as the nuclear export protein (NEP).

Replication of the Viral Genome

Transcription of the viral mRNAs and replication of the viral genome both occur in the nucleus of infected cells. Initiation of viral mRNA synthesis is primed by host cell RNA fragments containing an m⁷GpppXm cap structure (129, 130). These are generated from cellular pol II mRNA transcripts owing to an endonuclease activity provided by the viral polymerase. The host-derived fragments are between 10 to 13 nucleotides in length and do not hydrogen bond to the vRNAs. Template-directed extension of the primers proceeds from a G residue complementary to the C residue at position 2 of the vRNA 3' end. Termination of mRNA synthesis occurs due to a polyadenylation (poly-A) signal containing 5 to 7 U residues near the vRNA 5' end.

The switch from mRNA transcription to replication of antigenomic template RNAs and genomic vRNAs occurs later in infection, as it requires synthesis of

viral proteins (6, 58). It is thought that unprimed replication of cRNAs and vRNAs, as well as antitermination at the poly-A signal sequences, are dependent on the presence of newly synthesized soluble NP protein that has been imported back into the nucleus (9, 147). Encapsidated vRNAs are then exported from the nucleus and migrate to the plasma membrane where virion assembly occurs.

Packaging of the Gene Segments During Assembly

In theory, the number of possible reassortant viruses containing eight segments is 256, but in practice this is not observed. Although there are numerous examples in which particular gene segments are segregated randomly as a result of mixed infection, structural or functional constraints imposed on the gene products often result in the co-segregation of two or more segments. A number of genetic studies have indicated that functional cooperativity exists among subsets of viral proteins such as those of the polymerase complex, NP and M, and HA and NA (99). As an example, a balance between HA receptor binding and NA receptor destroying functions is clearly required for optimal replication. Drugs that inhibit neuraminidase function select for mutants not only in the active site of NA where the drug binds, but also in the region of the HA receptor binding site (104). In another example involving a mutant of A/WSN/33 virus containing a deletion in the NA stalk region that does not grow well in eggs, mutants were selected that fell into two categories. Half of these selected for insertions in the NA stalk and the other half involved mutations in HA that decreased sialic acid binding affinity (106). Glycosylation of HA near its receptor binding site and truncation of the NA stalk also combine to influence avian influenza virus replication (4). There are also examples in which the HA and M gene segments require functional compatibility between the HA fusion pH and M2 proton channel properties for efficient replication (50, 51).

The question of how influenza gene segments are incorporated into virions has been the subject of speculation for many years. The two predominant hypotheses involve either random packaging of gene segments or the existence of defined packaging signals for selectively including the different RNPs into virus particles. The true nature of gene segment packaging may lie between the most extreme interpretations of these postulates and involve aspects of each.

If packaging is completely random, the average virion would require incorporation of ten segments or more to ensure that the eight individual segments are present in the appropriate percentage of progeny viruses (37, 86). The pleiomorphic nature of virus morphology indicates that there should not be any strict physical limitations on the number of segments that can be incorporated into virions. Random packaging would likely lead to a high proportion of noninfectious virions being generated, and particle-to-infectivity ratios for influenza viruses have been estimated at around 10:1 (32). However, this is not significantly higher than some estimates for RNA viruses with nonsegmented genomes. Influenza viruses are potentially capable of incorporating more than eight segments. In experiments on reassortant

viruses generated by mixed infection of a ts mutant of A/chicken/Rostock/34 and A/chicken/Germany/49, Scholtissek et al. (141) showed that influenza "partial heterozygotes" containing both parental genes for either segment 3 or segment 6 could be maintained during plaque-to-plaque passages. Using reverse genetics, Enami et al. (37) demonstrated that a virus with a ts lesion in the NS1 gene could be complemented with an additional gene segment when grown at nonpermissive temperature. The segment with the NS1 mutation was required for providing NS2 function, whereas the ninth segment was required to express wild-type NS1. There are also numerous studies showing that artificial gene segments encoding reporter genes such as chloramphenicol acetyltransferase (CAT) or green fluorescent protein (GFP) can incorporate into infectious particles as a ninth segment, although the extra segment is usually lost following a few rounds of replication.

Several studies have compared the levels of vRNAs in infected cells with levels in budded virions, but the results of these fail to provide definitive conclusions. Hybridization studies by Smith & Hay (152) showed that in released particles of A/chicken/Rostock/34 virus the eight gene segments existed in equimolar amounts, whereas in infected cells these levels could be variable. However, in a similar study, Enami et al. (35) concluded that in cells infected with A/WSN/34 virus the vRNAs were synthesized coordinately and at equimolar levels. Studies on mutants with reduced levels of vRNA synthesis of individual segments are also inconclusive. A transfec tant influenza A virus containing the NA gene flanked by noncoding sequences derived from an influenza B virus NS segment was generated by reverse genetics (110), and this virus showed reduced levels of this particular vRNA both in infected cells and virions. However, in other studies on transfec tant viruses with mutations in the NA gene flanking sequences (12), wild-type levels of the segments were detected in virions, despite the observation that the relative amounts of these vRNAs were reduced in infected cells. One caveat to the latter two studies is that potential packaging signals may have been affected by the changes to the gene segment flanking regions.

The arguments for packaging signals include studies on influenza DI particles. These contain deletions in specific gene segments in which coding sequences have been removed and sometimes rearranged, but terminal regions are unaffected. Although they do not encode functional proteins, such segments can nonetheless be incorporated into virus particles. In several (but not all) examples when the truncated segment is incorporated into virions, it specifically interferes with the incorporation of the wild-type segment from which it was derived (2, 33, 112).

Studies on NA deletion mutants selected for growth in the presence of exogenously added neuraminidase, or on cells with depleted cell-surface sialic acid density, suggest that specific packaging signals may exist at terminal sequences of the segment. Liu & Air (93) passaged influenza in medium containing antibodies specific for the viral NA and soluble neuraminidase derived from the bacterium *Micromonosporum viridifaciens* and obtained mutants with large deletions in the gene segment that encodes the viral NA. Although these deletions eliminated the protein coding regions responsible for enzymatic activity, truncated gene segments

were maintained upon serial passage. The truncated NA gene segments of mutants selected in this manner contained the noncoding sequences at their 3' and 5' termini (172). In another study, mutant MDCK cell lines were generated by growth in the presence of lectins specific for sialic acid-containing influenza virus receptors. The mutant cell lines that were generated contained significantly reduced levels of cell-surface sialic acid, and virus propagation on such lines was not dependent on NA (72). Similar to the results of Liu & Air (1993), viruses passaged on such mutant MDCK cells gave rise to mutants with truncated NA gene segments containing internal deletions that eliminated NA enzymatic activity, but retained the terminal regions of the segment. In the above examples, the sequences retained at the vRNA 3' end minimally encode the short NA cytoplasmic tail and the hydrophobic segment that serves as both signal sequence and transmembrane anchor domain for the glycoprotein. It is possible that the small protein coding regions are maintained because the truncated proteins serve some function not related to NA activity, such as facilitating virus assembly. Infectious influenza viruses containing deletions of the six-residue cytoplasmic tail have been rescued by reverse genetics, but these replicate with reduced efficiency relative to wild type, and have altered morphology (45, 105). Alternatively, nucleotide sequences at the gene segment termini may contain specific packaging signals that cause the eight individual segments to be selectively incorporated into assembled virions in roughly equimolar quantities. The advent of improved reverse genetics techniques should allow this possibility to be addressed in the near future using reporter genes with alternative end sequences.

MUTANTS

Much of what is known about the functions provided by the individual gene products of influenza results from analyses of mutant viruses. Influenza, like other RNA viruses, displays a high mutation rate due to the error-prone nature of the viral polymerase, so mutant viruses are easy to isolate. Studies have been carried out on naturally occurring variants, spontaneous mutants derived in the laboratory, chemically or physically induced mutants, and mutants selected for a particular phenotype such as host range, drug resistance, or antigenicity. A selection of these, and the functional information obtained from their study, are described below.

Temperature-Sensitive Mutants

In the late 1960s and early 1970s, a large collection of influenza virus temperature-sensitive (ts) mutants, derived from differing strains, was generated in several laboratories worldwide [reviewed in (99)]. Such ts mutants were selected for growth at a permissive temperature (usually between 31°C and 36°C, depending on the study), but were significantly inhibited for replication at a higher nonpermissive temperature (usually between 38°C and 42°C). Simpson & Hirst (149) showed that pair-wise crosses of ts mutants at nonpermissive temperature could give rise

to non-ts viruses at frequencies much higher than standard revertions were observed. A series of crosses allowed them to identify five independent complementation groups with their mutants. Similar results with ts mutants were obtained in several other laboratories, and taken cumulatively, it was possible to identify eight separate complementation groups (99). By crossing parental strains with gene segments exhibiting different migration patterns on polyacrylamide gels and using various techniques for selecting reassortants of interest, it was possible to assign each complementation group to an individual gene segment. In some cases the function could be defined based on studies of these ts mutants and in others the results were ambiguous. Here we offer a few examples in which the phenotypes of given ts mutants led to insights on the functions specified by particular gene segments.

For example, studies on segment 1 mutants were useful for identifying the PB2 subunit of the polymerase as the viral component involved in binding to the m⁷GpppXm cap structures that are needed to prime viral mRNA synthesis. UV-crosslinking studies of purified polymerase complexes with radiolabeled cap structures showed that the PB2 subunit of wild-type virus could be labeled, but that segment 1 mutants of A/WSN/34 virus (WSN) were inhibited for cap binding function in a temperature-dependent fashion (161). The role for PB2 in cap binding is consistent with results obtained with an A/chicken/Rostock/34 (Rostock virus) mutant that was negative for in vitro transcriptase activity at nonpermissive temperature when rabbit globin RNA was used as primer, but displayed activity when the complementary dinucleotide ApG was used as primer (117). Several other segment 1 mutants also showed defects in RNA polymerase activity in vitro and in infected cells (47, 85, 109, 139, 158).

In vitro transcription assays using a ts mutant of segment 2, which encodes the PB1 protein, suggest that it may be involved in the initiation of transcription (108). This is consistent with transcription assays using radiolabeled nucleoside triphosphates, which showed that the first residue added to the primer bound to the PB1 subunit (68, 161). Assays on infected cells with a WSN PB1 ts mutant suggested that it plays a role in cRNA synthesis as well (85, 122). PB1 contains sequence motifs that are conserved among viral RNA polymerases (131), and mutagenesis studies show that these are required for vRNA synthesis (14), suggesting that PB1 may constitute the catalytic subunit for polymerization. PB1 also contains the nuclease activity required for capped primer formation (89).

Neuraminidase ts mutants provide one of the best examples in which the early studies clearly indicated the protein function. EM studies on WSN virus ts mutants showed that at nonpermissive temperature intact virus particles were produced at the plasma membrane of infected cells, but these aggregated and were inefficiently released (124). When bacterial NA was present during infection, no aggregation occurred and wild-type levels of HA activity was detectable in the infected-cell supernatants. Without functional NA activity to remove sialic acid from the viral and infected-cell surfaces, the interactions between HA receptor binding sites and sialoglycoconjugates prevented virus release.

A study on reassortants of a cold-adapted variant of A/Ann Arbor/6/60 virus used in the development of a live attenuated vaccine (98) provided results consistent with the view that M1 functions in virus assembly (120). At nonpermissive temperature this mutant was capable of synthesizing all viral proteins, but the M1 protein inefficiently associated with the plasma membrane resulting in the reduced production of virus particles. In another study, a ts M1 mutation of A/WSN/33 virus led to nuclear retention of the M1 at nonpermissive temperature (133). This did not affect the capacity for viral RNPs to exit the nucleus, but particle formation was significantly reduced.

Temperature-sensitive mutants of segment 8 have been identified, most of them with changes in the NS1 coding region. These mutants exhibit a variety of effects at nonpermissive temperature. Most display reduced synthesis of the M1, and in some cases the levels of HA, NS proteins, and vRNAs are also reduced [Reference (96) and citations within]. This is consistent with the multifunctional nature of the NS1 protein, which is involved in the regulation of both mRNA splicing and translation (87), as well as being an inhibitor of interferon-mediated antiviral responses (43, 174).

Antibody-Resistant Mutants

Antibodies that neutralize virus infectivity are directed against the surface glycoproteins, in particular the HA. As a consequence, the antigenic properties of the HA and NA change from one year to the next as the viruses evolve to evade existing human immunity. A number of studies have identified and characterized the binding sites for neutralizing antibodies, and much of what is known derives from the analysis of monoclonal antibody-resistant mutants (46). Sequencing studies on mutants selected for growth in the presence of anti-HA neutralizing monoclonal antibodies show that they map to regions on the surface of the globular membrane distal domains of the structure (167). The X-ray crystal structures of the HAs of escape mutants show that only localized changes occur, indicating that the mutated residues reside within the antibody footprints (150). For the HA of the H3 subtype virus A/Aichi/2/68, such studies led to the description of five antigenic regions of the molecule (designated A to E), and the location of the region at which resistant mutants were selected reflected the area of the molecule at which the antibodies were thought to bind based on EM studies of HA-antibody complexes (170). These interpretations have subsequently been confirmed by X-ray crystallography studies on such complexes (16, 40). Sequence changes in antigenic mutants selected by monoclonal antibodies against the H1 subtype have been interpreted similarly (21). The locations of the antigenic regions identified in studies on neutralizing anti-HA monoclonal antibodies are representative of the distribution on the structure of the sites that evolve during antigenic drift. The observation that these sites are proximal to the relatively conserved receptor binding pocket of HA, in conjunction with the structural data on HA-antibody complexes, indicates that interference with virus attachment to host cells

may constitute the principal mechanism for antibody-mediated virus neutralization (15).

The selection of escape mutants with monoclonal antibodies directed against the NA also allowed for the identification of the residues where changes were detected (1, 164). As with HA, NA mutations resulting in the loss of reactivity were shown to reside within the antibody footprints (119). The location of such mutations at regions of the NA structure adjacent to the enzyme active site (28) suggests that the antibodies act either by blocking access to the site or by distorting the catalytic domain (164).

Drug-Resistant Mutants

The observation that amantadine (1-aminoadamantane hydrochloride) exhibits anti-influenza activity was first made in the 1960s (30), and it was the first compound made available for clinical use against the virus. Amantadine, and the related compound rimantadine (α -methyl-1-adamantane methylamine hydrochloride), act at the level of virus entry by two separate mechanisms. At concentrations of 100 μ M or greater, *in vitro*, they have broad antiviral activity due to their properties as weak bases, which raises the pH of endosomes to mitigate against the pH-activated HA conformational changes required for membrane fusion (29). Mutants selected under these conditions contain substitutions in HA at various locations in the structure, all of which destabilized the protein as deduced by the elevated pH of fusion each displayed. At concentrations of 5 μ M or less these compounds exhibit specific activity against several strains of influenza A viruses, but have no effect on replication of influenza B or influenza C viruses. For many years the mode of action was unclear. In most strains it appeared to be at the stage of virus uncoating early in the replication cycle (18, 75). However, in certain strains of H5 and H7 subtype viruses with HAs that are cleaved intracellularly, an effect in the latter stages of infection was also observed (56). Experiments on reassortant viruses isolated following mixed infection of sensitive and resistant virus strains demonstrated that amantadine was operating on the M gene segment (57, 95). Subsequently, drug-resistant mutants were characterized, and it was found that they contained changes in the transmembrane domain of the M2 protein (59).

Several observations prompted speculation that M2 had a function in modulating pH gradients across membranes. With viruses containing HAs that are cleaved intracellularly, it was shown that the presence of amantadine late in infection caused the HAs to be expressed in the low pH conformation, but that this could be circumvented by the addition of agents that elevate the pH of intracellular compartments (159). It was also found that M2 can form tetramers (66, 160), which can be modeled as a four-helix channel in which residues selected for amantadine resistance oriented toward the interior. Furthermore, an amantadine-resistant Rostock virus mutant was isolated that had no changes in M2, but contained a mutation in the HA that rendered it much more stable to acid pH (154). The concept of M2 as a channel protein was subsequently confirmed by electrophysiological

experiments (127), and it was determined that the M2 channel activity is selective for protons (24).

Current models for the mode of action of amantadine reason that, during entry, as endosomes are acidified the M2 allows for the concurrent acidification of the virus interior. This is thought to be required for the dissociation of the viral nucleocapsids from the M1 protein (175), which has been proposed to be important for the subsequent transport of nucleocapsids into the host cell nucleus (18, 101). The function of M2 late in infection is thought to involve the removal of protons from trans-Golgi or post-Golgi vesicles during transport of the viral glycoproteins to the cell surface. This is particularly important for HAs that are cleaved in the Golgi apparatus, as they are more susceptible to acidic conditions that could lead to premature conformational changes.

A number of compounds targeting the NA have been developed with the rationale that if they bind to the active site of NA more tightly than sialic acid, they may have antiviral activity (162). In clinical trials the compounds zanamivir and oseltamivir have proven effective both for prophylactic and therapeutic purposes [reviewed in (53)], although when given at the onset of symptoms they generally reduce the length of illness by only about one day. The drugs appear to act as inhibitors of virus release and dissemination (52) as anticipated based on the knowledge of NA function. Mutants resistant to these drugs are not selected as readily as with amantadine; however, a number of examples have been characterized [reviewed in (104)]. Although mutants with changes in the NA were among those reported, those with changes in the HA were actually more abundant. As expected, the NA mutants contained changes in the active site of the enzyme that involved either residues that play a role in catalysis, or residues involved in structural stability. The HA changes were primarily at residues in, or in close proximity to, the sialic acid–receptor binding pocket, and presumably they alter the HA binding properties. These observations substantiate the concept of interplay between the HA and NA—the loss of NA function can be compensated for by a reduction in binding affinity by the HA. As such, the virus particles can elute from cells despite the lack of NA function caused by the inhibitors.

Receptor-Binding Mutants

Differences in sialic acid binding specificity of influenza virus HAs primarily involve their distinction between sialic acid in $\alpha(2,3)$ - and $\alpha(2,6)$ -linkages to galactose on carbohydrate side chains. Most HAs of avian and equine influenza viruses preferentially recognize receptors containing the $\alpha(2,3)$ linkage, and those of human viruses the $\alpha(2,6)$ linkage. However, these generalizations should not be considered an “all-or-none” phenomenon, as various residues may play a role in specificity and mutants can potentially display intermediate phenotypes. HA mutants of human viruses that recognize $\alpha(2,3)$ -linkages can be selected by growth in the presence of glycoproteins rich in $\alpha(2,6)$ -linked sialic acids. These mutants defined the location of the receptor binding site on the HA molecule and sequence, and

X-ray crystallographic analyses have indicated the importance of HA1 residue 226 in the site for the differing receptor specificities of viruses from different species (134). The importance for receptor binding generally of conserved residues that form the receptor-binding site has been confirmed by site-specific mutagenesis of expressed HAs and the study of mutants generated by reverse genetics (100).

RNA RECOMBINATION

For influenza viruses there have been relatively few documented cases of true RNA recombination, as opposed to gene segment reassortment. Early sequencing studies on the RNA genomes of influenza revealed examples of inverted sequences within gene segments (38), and that the generation of DI particles resulted from recombination events within or between gene segments (39, 74, 113). In another study, Rohde & Scholtissek (135) observed that following mixed infection of two viral strains, one reassortant contained an NP segment with sequences derived from each parental virus as a result of a recombination event.

There are also examples of viruses using nonhomologous recombination to acquire pathogenic traits relating to HA cleavage, which is required to activate membrane fusion potential (see below). In one example, the nonpathogenic virus A/turkey/Oregon/71 was selected for growth in tissue culture in the absence of a protease to cleave HA. This resulted in the acquisition of a 54-nucleotide insertion derived from 28S ribosomal RNA at the region encoding the HA cleavage site that could be cleaved by intracellular proteases, and the resulting virus was more pathogenic for chickens than the parental virus (81). In a similar study with mutants of A/seal/Massachusetts/1/80 virus, a 20-amino acid insertion of residues derived from the viral NP was found at the cleavage site (121).

Several examples of RNA recombination were also demonstrated in experiments with viruses generated by reverse genetics, including one example of a mosaic gene segment being generated in which sequences derived from the M, PB1, and NA genes of A/PR/8/34 virus were incorporated into a bicistronic gene segment encoding A/WSN/33 NA and a CAT reporter gene (11).

A more recent study illustrated that the frequency at which non-homologous recombination can occur during influenza replication may be higher than once appreciated (106). This study utilized a mutant of WSN virus containing a 24-residue deletion in the stalk region of the viral NA. On MDCK cells this mutant virus grows to titers equivalent to WT, but in embryonated chicken eggs it is severely debilitated. Following continuous passage of the mutant in eggs, 10 independent clones that grew efficiently were isolated. Five of these contained insertions in the NA stalk region, ranging from 10 to 22 amino acids, which were derived from coding sequences for either the PB1, PB2, or the NP proteins. The other five clones displayed reduced HA receptor-binding activity to compensate for the reduction of NA function.

Although examples of true RNA recombination of influenza viruses in nature have been rare, a recent phylogenetic analysis of the HA gene from viruses thought

to be responsible for the pandemic of 1918–1919 prompted the investigators to suggest that these viruses may have been HA recombinants (48). They speculated that the HA2 sequences, which compose most of the stalk region of the molecule, may have derived from a human-lineage influenza, whereas the HA1 globular head domain of the molecule, which contains the receptor binding site and antigenic regions, came from swine viruses. However, an alternative interpretation of the data has also been proposed (168).

REVERSE GENETICS

The genetic manipulation of negative strand viruses such as influenza proved to be a challenge, as neither the genomic RNA (vRNA) nor the antigenomic RNA (cRNA) are infectious as naked RNA. For transcription and replication to occur these RNAs must be encapsidated by the NP protein and associated with the proteins of the polymerase complex, PB1, PB2, and PA. In the late 1980s, a major breakthrough for reverse genetics using negative-strand viruses came through studies on influenza virus by Palese and colleagues. They showed that purified NP and polymerase proteins could be used to reconstitute replication-competent *in vitro* transcribed RNAs (97), and these techniques soon led to the rescue of infectious viruses containing specified mutations (36). The generation of such viruses involved infection of cells by a “helper virus,” transfection of the cells with *in vitro*-generated mutant RNPs, and selection of viruses containing the mutant gene segment away from the pool of viruses with the homologous segment derived from the helper. It subsequently became possible to apply the reverse genetics technology to a broad range of topics including functional studies on several of the viral proteins, examination of the requirements of the noncoding sequences for transcription and replication, the expression of foreign antigens, and the use of manipulated viruses for vaccine studies (44, 114).

The principal limitation on the infection-transfection system was the requirement for a highly efficient selection system. Selection systems based on properties such as host range, antibody reactivity, and drug susceptibility eventually led to the development of rescue systems for six of the eight gene segments, but debilitated viruses could be difficult to rescue, and often the choice of viral strains that were amenable for study was limited. Over several years, many laboratories developed variations of the techniques for virus rescue in attempts to improve efficiency, but none of these eliminated the requirement for helper virus and selection. However, one such alternative developed by Hobom and colleagues ultimately proved to be a significant step forward, as it circumvented the requirement for generating the RNPs *in vitro* (116). This approach involved the use of cDNAs containing viral gene segments flanked by RNA polymerase I (pol I) promoter and terminator sequences. RNA pol I normally transcribes ribosomal RNAs, which are not capped and contain no signals for 3' polyadenylation. The pol I promoter and terminator direct defined 5' transcription start and 3' stop signals, so it was possible to use these to generate influenza gene segment RNAs with the correct termini. Pleschka

et al. (128) then demonstrated the utility of employing constructs such as these for making infectious viruses using the helper virus system.

In 1999, the long-term goal of generating influenza virus entirely from plasmid DNAs was realized (41, 115). In the system developed by Kawaoka and colleagues (115), the eight gene segments of WSN virus were each cloned into plasmids between the human pol I promoter and mouse pol I terminator and these were transfected into human 293T cells along with plasmids encoding the nine influenza structural proteins. Remarkably, within two days in excess of 10^7 plaque-forming units per ml. were recovered from the transfected cell supernatants, and more recently the efficiency has been improved approximately tenfold (114). Using a similar plasmid-only virus rescue system that utilized a pol I promoter to generate 5' RNA ends and hepatitis delta virus ribozyme sequences to generate the 3' termini of the gene segments, Fodor et al. (41) also demonstrated the rescue of infectious viruses. Subsequently, the pol I system has been modified by Hoffmann et al. (65) to reduce the number of plasmids required to eight. In this system the gene segment RNAs flanked by pol I promoter and terminator sequences are in turn flanked by a CMV promoter (pol II) and polyadenylation signals in the opposite orientation such that viral mRNAs can be transcribed from the same plasmid.

ANTIGENICITY

The genetic properties and ecological diversity of influenza A make it a classic example of a re-emerging virus. Influenza A viruses are renowned for their capacity to cause epidemics on a nearly annual basis due to the continuously evolving nature of their surface glycoproteins, referred to as antigenic drift. At unpredictable intervals viruses with completely different surface antigens are introduced into humans. As large segments of the population have little or no immunity to these strains, they cause global pandemics. This is known as antigenic shift.

Antigenic Shift

Serologically, 15 nonoverlapping subtypes of HA and 9 subtypes of NA have been identified. All of these occur in viruses that circulate in aquatic birds, and these species serve as the natural reservoir for all influenza A viruses (169). Until recently, only H1N1, H2N2, and H3N2 viruses were known to have circulated extensively in humans. In 1918 and 1919, H1N1 viruses were responsible for the pandemic of "Spanish flu." This was the worst outbreak of infectious disease in human history, causing an estimated 20 to 40 million deaths. Strains related to the 1918 viruses continued to circulate in humans until 1957, when they were replaced by H2N2 subtype "Asian" strains resulting in the next pandemic. In 1968, another antigenic shift occurred with the emergence of H3N2 subtypes of "Hong Kong flu." In this case the HA, but not the NA, was antigenically novel to humans. The fact that a pandemic resulted illustrates the concept that neutralizing antibody responses targeting the HA provide the key component for protection against

influenza, although residual immunity to the NA may have reduced the severity of disease resulting from the 1968 viruses.

Descendants of these H3N2 viruses have continued to circulate in humans to this day. However, in 1977 H1N1 subtype viruses that were virtually identical to strains that had previously circulated in 1950 re-emerged in humans (80, 111, 143). The source of the viruses that re-emerged in 1977 is unknown, but it is possible that they had been preserved in a frozen state. Unlike the situation with H2N2 viruses in 1957 and H3N2 viruses in 1968, the 1977 H1N1 strains did not displace the H3N2 viruses. For the past 25 years H3N2 and H1N1 viruses have co-circulated in humans. This has offered the opportunity for these human viruses of different subtypes to reassort with one another (173), and within the past year a number of H1N2 subtype viruses have been isolated from humans (Y. P. Lin & A. Hay, personal communication).

In theory, there are several potential routes by which viruses with novel surface antigens could be introduced into humans. An avian virus could transmit directly to humans, as appears to have been the case with the 1997 H5N1 viruses described below, and with the avian H9N2 subtype viruses that transmitted to humans in 1999 (91). It is also possible that avian-like viruses could infect humans following an intermediate period in an alternative host, as a number of influenza viruses have also been isolated from other mammals. For example, H1N1, H3N2, H9N2, H1N7, and H1N2 subtypes have all been isolated from pigs, and H3N8 and H7N7 subtypes from horses. There have also been sporadic examples of viruses of various subtypes being isolated from seals, whales, and minks (165). The viruses that caused the pandemics of 1957 and 1968 were derived from the reassortment of avian and human strains (77, 144). In 1957, the HA, NA, and PB1 gene segments came from an avian virus and the other segments were obtained from the human H1N1 viruses that were circulating at the time. The H3N2 viruses that emerged in 1968 resulted from the replacement of the HA and PB1 gene segments of circulating human H2N2 viruses with their counterparts from an avian source. The host species in which these reassortment events took place is not known. Avian viruses generally do not replicate well in humans (3), and vice versa (62), so it is possible that reassortment took place in an intermediate host. Pigs have been proposed as a potential "mixing vessel" for reassortment (140), and both avian and human strains can replicate in these hosts (61, 82). One reason for this may be that cells of the pig trachea, where influenza replication occurs, contain the $\alpha(2,3)$ -linked sialic acid receptors preferred by avian viruses, as well as those with the $\alpha(2,6)$ linkage favored by human strains (73).

Antigenic Drift

In aquatic birds that provide the natural reservoir for influenza A viruses, phylogenetic analyses show the viruses to be virtually in evolutionary stasis (165). However, when antigenically novel strains are introduced into humans they rapidly and continuously evolve, presumably due to immune selection and various factors that

may be involved in adapting to a new host. The glycoproteins in particular exhibit rapid rates of evolution. For the HA the rates of evolution approximate 6.7×10^{-3} substitutions per nucleotide per year and for the NA, 3.2×10^{-3} substitutions per nucleotide per year (151). In the HA most of these occur in the HA1 domain at antigenic sites described above, as a result of immune selection. The genetic drift of the glycoproteins necessitates constant surveillance in order to monitor the antigenicity of circulating strains such that vaccines can be updated when necessary.

If one assumes that humans normally generate polyvalent antibody responses to a given antigen, a question that arises regarding drift concerns the reason that individuals can be re-infected with a given subtype in subsequent years, and how the same subtype can continue to circulate for several decades. One clue, based on the analysis of postinfection human sera, indicates that antibody responses are composed of a limited antibody repertoire (163). As a consequence, only a limited number of changes in the viral glycoproteins may be required to evade the immune response generated by any particular individual to a previously circulating virus.

PATHOGENICITY

For certain avian strains of H5 and H7 subtype viruses the cleavage properties displayed by the HA are the most notable determinant of virus pathogenicity. However, it is also clear that several, if not all eight, gene segments can contribute to the pathogenic phenotype of a virus, depending on considerations such as the genetic background of the virus, the host, and the route of infection.

HA Cleavage as a Major Determinant of Pathogenicity

HA is synthesized as a precursor polypeptide (22) that must be cleaved into the disulfide linked subunits HA1 and HA2 in order to activate membrane fusion function and virus infectivity (84, 88). For the HAs of mammalian and most avian influenza A viruses this cleavage activation step occurs only after the glycoprotein has been transported to the plasma membrane. This restricts the sites of replication in infected animals to organs or tissues where appropriate extracellular proteases are present. In contrast, there are a number of examples of highly pathogenic avian virus strains, in particular among H5 and H7 subtype viruses, which can cause systemic infections in hosts such as chickens and turkeys. The HAs of these viruses are cleaved intracellularly, in the Golgi apparatus, by subtilisin-like proteases such as furin or PC6 (67, 155). These proteases are present in nearly all cell types, and this alleviates one of the restrictions on the potential sites for multicycle replication in the host and facilitates systemic spread of the virus.

In the HA precursor, the cleavage site exists as a loop structure that extends out from the surface of the molecule (22). In nonpathogenic viruses the HA generally contains a single arginine residue at the site of cleavage, which is recognized by extracellular trypsin-like proteases. The HAs of highly pathogenic viruses are

often characterized by insertions of additional basic residues at the site of cleavage (83, 153, 166). This serves two functions regarding protease recognition. It extends the site of cleavage further from the surface of the trimer, presumably making it more accessible for activating proteases, and it generates sequence motifs that match or closely resemble the consensus furin recognition sequence R-X-R/K-R. Another factor that can influence cleavage involves the presence or absence of carbohydrate attachment sites adjacent to the cleavage loop. There are examples in which mutations that eliminate such glycosylation sites lead to acquisition of the highly pathogenic phenotype, most likely as the result of an increase in accessibility of the cleavage loop (31, 78, 79).

Of the viruses that have circulated in humans over the past century, there have been no reported examples of strains that contain HA cleavage site features associated with highly pathogenic viruses. However, in 1997 pathogenic avian H5N1 subtype viruses crossed the species barrier and caused a limited outbreak in humans in Hong Kong. Fortunately, these viruses did not develop the capacity for efficient human-to-human transmission, but of the 18 diagnosed cases, 6 were fatal. All of the viruses isolated from H5N1-infected humans were found to contain polybasic sequences at the HA cleavage site that provide furin recognition motifs (10, 27, 148, 156, 157). The viruses isolated were closely related, but in studies using mouse models, nearly all isolates segregated into either high- or low-pathogenicity phenotypes (34, 42, 54, 94, 118). One sequencing study showed that among viral proteins of these isolates, five mutations in four different proteins other than HA correlated with high pathogenicity (76). Two of these were in the PB1 protein, and one each in the PB2, NA, and M1 proteins. An alternative approach using reverse genetics implicated a single mutation in the PB2 protein as the critical determinant of pathogenicity among these isolates in mice, although a notable effect due to heterogeneity of a residue in the HA receptor binding site was also observed (55). Interestingly, the PB2 mutation identified in this study was distinct from the PB2 change observed in the sequencing study. The viruses were not highly pathogenic unless the polybasic sequence at the HA cleavage site was present, regardless of the other mutations, indicating that it is necessary, but not sufficient, for full manifestation of the phenotype.

Multifactorial Nature of Pathogenicity

Examples of studies suggesting that pathogenicity is a polygenic trait date from the 1950s. In experiments that involved mixed infection of the mouse neurovirulent strains WSN or NWS with avirulent strains such as A/Melbourne/35, it was observed that progeny viruses could be generated in which the virulence and antigenic properties were segregated, and that recombinant viruses with varying degrees of virulence could also be obtained (64, 92). In another example, Mayer et al. (102) showed with reciprocal recombinants of NWS virus (H1N1) and the avirulent virus A/Japan/305/57 (H2N2), that both H1N2 and H2N1 viruses were capable of lethally infecting mouse brains, suggesting that neither surface antigen

is the exclusive determinant of neurovirulence. Numerous other genetic studies on WSN and NWS strains of influenza have been carried out over the years to indicate that more than one gene influences virulence, and these have been reviewed extensively by Schlesinger et al. (138). However, studies on WSN using reverse genetics technology suggest that a glycosylation site and a C-terminal lysine residue (49, 90) of the viral NA allow it to sequester plasminogen and facilitate HA cleavage (49), and this may have a particular significance for the virulence of this strain. Thus, it seems that when gene segments harbor traits that are known to influence pathogenicity, the genetic background provided by other segments can play a significant role in modulating this phenotype.

Studies on reassortant avian viruses also provided evidence for the polygenic nature of pathogenicity and the complexity of this phenomenon. For example, the viruses A/chicken/Rostock/34 and A/turkey/England/63 both contain polybasic sequences at the HA1-HA2 cleavage site and are highly pathogenic for chickens. However, both nonpathogenic and pathogenic viruses were generated from these strains following co-infection of chick cells, and both HAs were represented among the nonpathogenic reassortants (137). In these viruses, it seemed that the mixing of segments encoding proteins of the polymerase complex was responsible for the loss of pathogenicity. In another study, Rostock virus reassortants were generated containing single gene segment replacements, in which the replaced segments were derived from a variety of different virus strains (142). Among these reassortant viruses, the capacity for modulation of pathogenesis was demonstrated for each of the seven gene segments analyzed.

Many viruses, including influenza, are capable of inducing apoptosis, and this property may contribute to the pathogenicity of these viruses. Different strains of influenza vary in their capacity to induce apoptosis, but detailed mechanisms on how or why this is the case are not yet clear. The viral NA (107, 146) and NS1 (145) proteins have both been implicated in triggering apoptosis, and recently it was reported that the PB1 gene can encode a reasonably well-conserved 87-residue protein from an alternative reading frame, which has the capacity to localize to the mitochondria and induce cell death (23).

INFLUENZA B AND INFLUENZA C VIRUSES

Influenza B and influenza C viruses are orthomyxoviruses that are related to influenza A virus, but intertypic reassortment among the three genera has not been demonstrated. Influenza B virus can cause disease symptoms similar to influenza A, and in some years is probably responsible for more illness than influenza A (169). In general, influenza C virus causes less severe respiratory illness, which rarely progresses to the lower respiratory tract. Although there have been examples of influenza B and C viruses transmitting to other hosts they are essentially human viruses, so unlike influenza A, they do not have a natural reservoir from which they can recruit antigenically novel surface antigens. This may be one factor involved in the different patterns of antigenic drift displayed by influenza B and C viruses.

by comparison to influenza A. Whereas influenza A viruses generally follow a single evolutionary lineage from one year to the next, antigenically diverse strains of influenza B and C viruses co-circulate (125).

With regard to genome structure, the major difference among influenza A, B, and C viruses pertains to the surface glycoproteins. Influenza A and B viruses have eight gene segments and contain separate attachment and receptor-destroying envelope glycoproteins (HA and NA), whereas influenza C virus has only seven gene segments, with a single glycoprotein, the HEF, providing both of these functions as well as membrane fusion activity. The structures of influenza A HA and influenza C HEF demonstrate that they are largely comparable, but with HEF a domain responsible for the receptor destroying esterase activity is inserted between the stalk region of the protein and the receptor binding domain (136). Other differences among the three genera involve the coding strategies for the gene segment encoding the NA and matrix proteins. Influenza B virus segment six, which encodes the NA, also encodes a protein that may be functionally similar to the influenza A M2 protein. This protein, designated NB, uses an alternative initiation codon to synthesize a 100-residue type III membrane protein that, like M2, is incorporated into virions (13, 17). In addition to the M1 protein, segment seven of influenza B virus also encodes a protein of unknown function, BM2, by utilizing a tandem cistron translational stop-start mechanism (70). Influenza C virus segment six encodes the proteins CM1 and CM2 that may be functionally similar to the influenza A M1 and M2 proteins. CM1 derives from a spliced message (171), whereas CM2 is the proteolytic product of a precursor protein that also produces a rapidly degraded membrane-bound polypeptide of unknown function (126).

Among the influenza A, B, and C viruses, proteins that appear to have similar functions have evolved strategically different mechanisms for their expression during virus replication. From an evolutionary standpoint, it will be interesting to analyze and compare the structural and functional properties of the proteins encoded by these viruses, to attempt to relate these to the various ecological niches that these viruses inhabit, and to understand how and why these viruses can establish themselves in a particular host while possibly maintaining the genetic means to transmit to another.

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Intracellular trafficking of influenza virus: clinical implications for molecular medicine

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The trafficking and processing steps that occur in cells that are infected with influenza virus play a crucial role in the outcome of infection. These steps are targets for new and future anti-viral drugs, and can affect the relative virulence of the virus and its ability to cause disease. The virus first binds to its host cell via specific sialic acid residues, which can control the species tropism of the virus. The internalisation of the virus, into the nucleus of the cell, is dependent on a low pH, and this process is therapeutically targeted by the drug amantadine. Following replication, the newly formed viral genomes leave the nucleus and assemble into infectious particles at the plasma membrane. The targeting and processing of the various viral components at this late stage of the infectious cycle can have a major effect on the ability of the virus to spread and cause disease in its host. Finally, the release of viruses is dependent on the enzyme neuraminidase (NA), and this function has recently been targeted by the NA inhibitors, a new generation of drugs against influenza virus.

Like all viruses, influenza has an intimate relationship with its host cell and, during the course of replication, it undergoes many important trafficking steps. A study of such intracellular trafficking has revealed many facets of the virus life cycle, and how the life cycle relates to the pathogenic properties of the virus. Some of these trafficking steps can be targeted by anti-viral drugs, which are used to treat influenza virus infections. The trafficking of influenza virus within its host cell, and how these events are related both to the pathogenic properties of the virus and to therapeutic treatments for viral infection have been discussed in this review.

Strategies for vaccination have not been covered; instead, readers are referred to Subbarao (Ref. 1) for more information on this topic.

Aetiology and pathogenesis of influenza viruses

In humans, influenza viruses are common pathogens of the upper respiratory tract, and seasonal epidemics affect 10–20% of the general population. However, the virus can also be deadly. It has been estimated that the now-infamous influenza pandemic of 1918–1919 killed 20–40 million people worldwide (Ref. 2). Influenza viruses infect humans and a wide variety of

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animals (Refs 3, 4). Other mammals that are susceptible to respiratory influenza infection include pigs, horses, mink, seals and whales. The virus also has gastrointestinal tropism in various bird species.

Influenza virus is a member of the *Orthomyxoviridae* family of RNA viruses, and is an enveloped virus (Fig. 1). Its genome consists of individual segments (or genes) of negative-sense RNA. There are three types of influenza viruses: A, B and C. Influenza A viruses are the most widespread and infect many animal species. Influenza B and C viruses were originally thought to cause disease only in humans; however, influenza B virus infection has recently been discovered in seals (Ref. 5). It is currently unclear whether this is a single transmission event or whether it implies a wider distribution of the virus.

Influenza virus subtypes are designated by a nomenclature that is based on their surface glycoproteins, namely haemagglutinin (HA or H) and neuraminidase (NA or N; also known as sialidase). The first human influenza viruses to be isolated during the 1930s were subsequently designated H1N1, based on their serological reaction. This group includes the viruses that are now known to have been present in the pandemic 1918 strain. In 1958, an antigenic shift resulted in the emergence of human H2N2 viruses and, in 1968, a shift to H3N2 viruses occurred in human populations. H3N2 viruses have remained the most prevalent in recent years (Ref. 6), but the re-emergence of H1N1 strains during the 1970s, most probably from a laboratory source, has resulted in co-circulating influenza H1N1 viruses. Influenza B viruses have not been given the same H and N designation and, in recent years, have represented a minor population of circulating viruses in humans. Influenza C viruses generally result in only mild respiratory illness and are much less studied.

The emergence of new influenza strains in the human population occurs via transmission from other animal species, most notably birds. Transmission to humans is most commonly thought to occur through an intermediate such as swine (Ref. 3). Typically, human and avian influenza viruses are quite different and are not infectious for both species. However, pigs can become infected with both types of viruses, and it has been proposed that they act as a 'mixing vessel' for the transmission of avian



Electron micrograph
of an influenza virus

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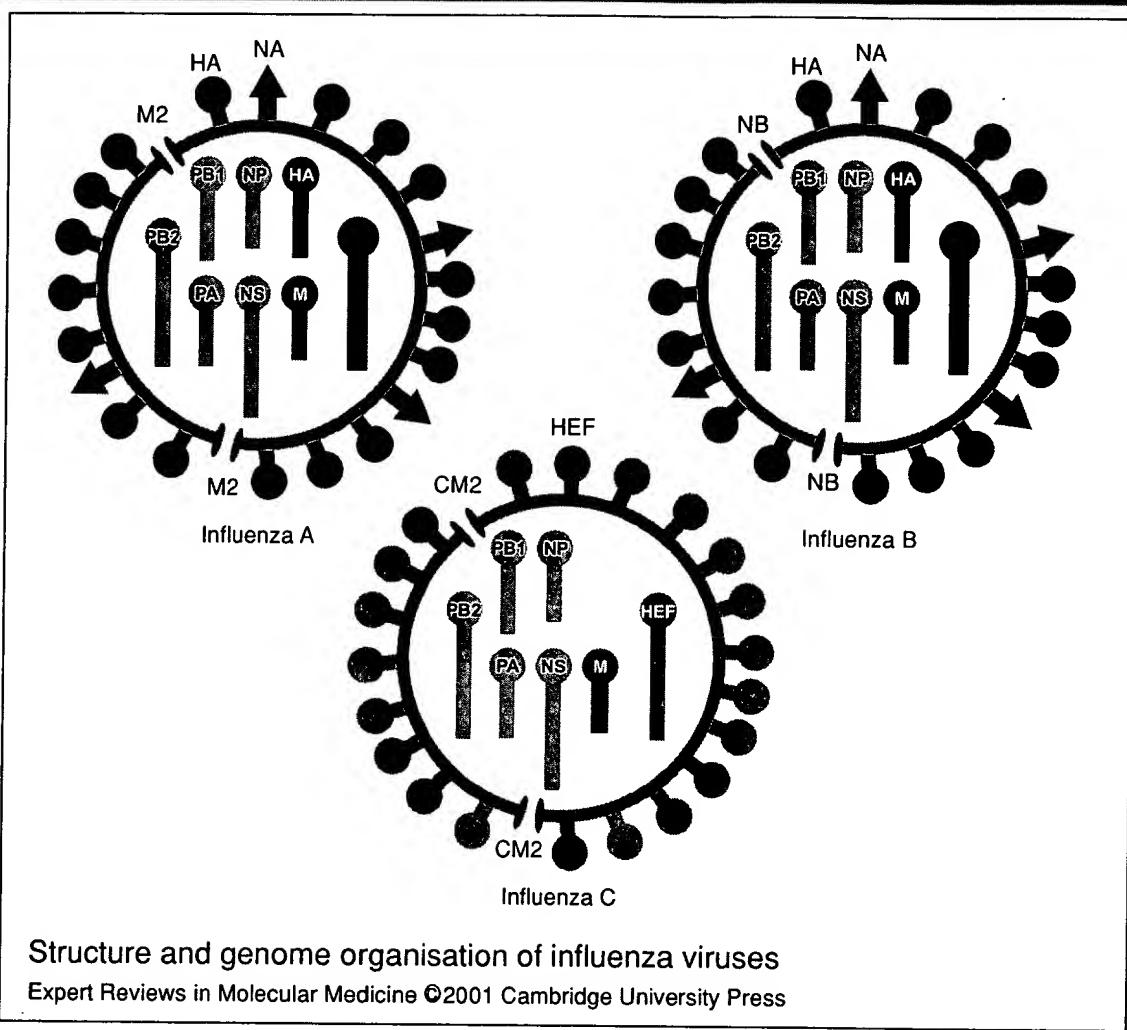
Figure 1. Electron micrograph of an influenza virus. This influenza virus (strain A/WSN/33) was purified from Madin-Darby bovine kidney (MDBK) cells and negative-stained with potassium phosphotungstic acid. (The figure was kindly provided by Elizabeth Wills, Cornell University, Ithaca, NY, USA) (fig001gwn).

influenza viruses to humans. Occasionally, direct avian–human transmission can occur with deadly results, as demonstrated by the emergence of the recent H5 'Hong Kong avian flu' during 1997–1998 (Ref. 7). Several instances of the transmission of this virus from domestic chickens to humans proved fatal, but no proven cases of human to human transmission occurred.

Structure of influenza viruses

Influenza viruses are enveloped and are pleiomorphic (i.e. they can vary their size and shape). Viruses isolated from cell culture are typically spherical, with a constant diameter of ~100 nm (see Fig. 1). However, the virus can also be filamentous; particles retain a constant diameter (of 100 nm) but vary in length (up to several micrometers; Ref. 8). Filamentous viruses, such as these, are likely to predominate in clinical situations, and to be important in natural infection by influenza virus (Ref. 9).

Influenza A viruses produce ten proteins from eight RNA segments (Refs 10, 11). The eight negative-sense RNAs are associated with



Structure and genome organisation of influenza viruses

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Figure 2. Structure and genome organisation of influenza viruses. The surface proteins of each virus and their respective genes are shown in colour (blue, red and green); other genes are shown in light grey. The interior proteins, namely the matrix protein (M1), the nucleoprotein (NP) and the polymerases are not shown. Influenza A and B viruses contain eight RNA segments (genes), whereas influenza C viruses contain only seven RNA segments. Influenza C viruses contain a single surface glycoprotein (the haemagglutinin-esterase-fusion, or HEF, glycoprotein; shown in light blue), which functionally replaces the two surface glycoproteins that are found in influenza A and B viruses, namely haemagglutinin and neuraminidase [HA (shown in dark blue) and NA (shown in red)]. The envelopes of the three viruses also contain different ion channels, which are encoded by either the M gene (i.e. M2 or CM2, shown as green ovals) or the NA gene (i.e. NB, shown as red ovals) (fig002gwn).

many copies of a nucleoprotein (NP) and a heterotrimeric polymerase, which form the viral ribonucleoproteins (vRNPs). Inside the virus, the vRNPs are surrounded by a shell of the matrix protein (M1). M1 links the vRNPs to the virus envelope, which contains the viral glycoproteins as well as the tetrameric M2 ion channel. Host-cell proteins are typically excluded from the mature virus particles. All influenza viruses have

similar internal components, but the constituents of their envelopes can differ markedly (see Fig. 2). Influenza B viruses have an alternative ion channel (NB) that is produced as an overlapping reading frame by alternative initiation from the gene encoding NA. Influenza C viruses have a single glycoprotein (the haemagglutinin-esterase-fusion, or HEF, glycoprotein) that functionally replaces both HA and NA. Influenza C viruses

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also differ in having an alternative ion channel (CM2) and a genome that consists of only seven segments of RNA, rather than eight. Influenza viruses also synthesise two proteins (NS1 and NS2) that were originally considered to be non-structural. However, NS2 is now believed to be part of the virus particle. During virus replication, the genes that encode M and NS are both spliced, accounting for the synthesis of two additional polypeptides.

Cell and tissue tropism of influenza viruses

In vivo, the principal cell types targeted by influenza viruses are the cells in the epithelial lining of the respiratory mucosa, which is a polarised epithelium; that is, it has distinct apical and basolateral surfaces (Refs 12, 13). When an aerosolised virus is inhaled, the virus encounters the apical (or outside) face of columnar epithelial cells. Following replication, virus is also released from the apical face of the cell into the airways of the respiratory tract. The lack of basolateral release generally precludes the systemic spread of influenza viruses in their host. Released viruses can spread from cell to cell, be exhaled and infect a new host. They can also be recognised by cells of the immune system, including alveolar macrophages (which engulf and destroy the virus) and circulating dendritic cells (which migrate out of the lung tissue and present viral antigens to T cells). In the laboratory, viruses are typically studied either in embryonated chicken eggs or in Madin-Darby canine kidney (MDCK) cells, both of which support the multi-cycle growth of influenza viruses (Ref. 14). Owing to the wide distribution of receptors, many other cell types can be infected by the virus, but some only through a single cycle of infection and without the spread of virus from cell to cell.

Virus binding, internalisation, trafficking and export

A single cycle of influenza virus infection in a typical cell is depicted in Figure 3. Briefly, the virus initially binds to its cell-surface receptor and is internalised into endosomes (cytoplasmic vesicles), where the low pH of the environment triggers virus fusion and uncoating. The uncoated vRNPs then enter the nucleus of the host cell for virus replication. Following virus replication, the vRNPs leave the nucleus and move to the plasma

membrane, where they associate with viral glycoproteins before final budding and release. Each of these transport events is considered below, in relationship to our current understanding of influenza virus pathogenesis and treatment.

Virus binding

It is well established that human influenza viruses bind to moieties that contain 5-N-acetyl neuraminic acid (sialic acid) on the surface of host cells; however, in the pig and horse, N-glycolyl neuraminic acids can be used. Binding to sialic acid occurs via a shallow depression near the membrane-distal tip of the HA glycoprotein. It is also well established that some viruses bind preferentially to terminal sialic acids containing α -(2,6) linkages, whereas others favour binding to α -(2,3)-linked sialic acid (Ref. 15). This receptor-binding specificity correlates with a specific amino acid at position 226 of HA. HAs that have leucine at position 226 selectively bind to α -(2,6) sialic acid and occur preferentially in human strains. However, HAs that have glutamine at position 226 are specific for α -(2,3) linkages, and occur mostly in avian and equine strains of the virus. Both α -(2,3)- and α -(2,6)-linked sialic acid occur in the trachea of swine – accounting for the ability of pigs to become infected with both avian and human strains. Thus, receptor binding is one of the initial determinants of pathogenicity, such that the specificity of receptor binding accounts for much of influenza's species tropism (i.e. the predilection of the virus to infect certain animal species and not others).

The cell-surface receptor for influenza viruses can apparently take the form of sialic acid linked to either glycoprotein or glycolipid. *In vitro*, viruses can bind to, and fuse with, synthetic lipid vesicles that contain only glycolipid (Ref. 16), suggesting that entry does not require a specific cellular protein as a receptor. However, the infection of desialylated cells has been reported recently, suggesting either the presence of sialic-acid-independent receptors or a multi-stage process (Ref. 17). *In vivo*, other factors may well be important for virus entry. In macrophages, recent evidence suggests that the viruses undergo an additional lectin-like interaction with the mannose receptors of the macrophages, following the initial interaction with sialic acid (Ref. 18). Whether other 'co-receptor-like' activities occur in other cell types remains to be determined.

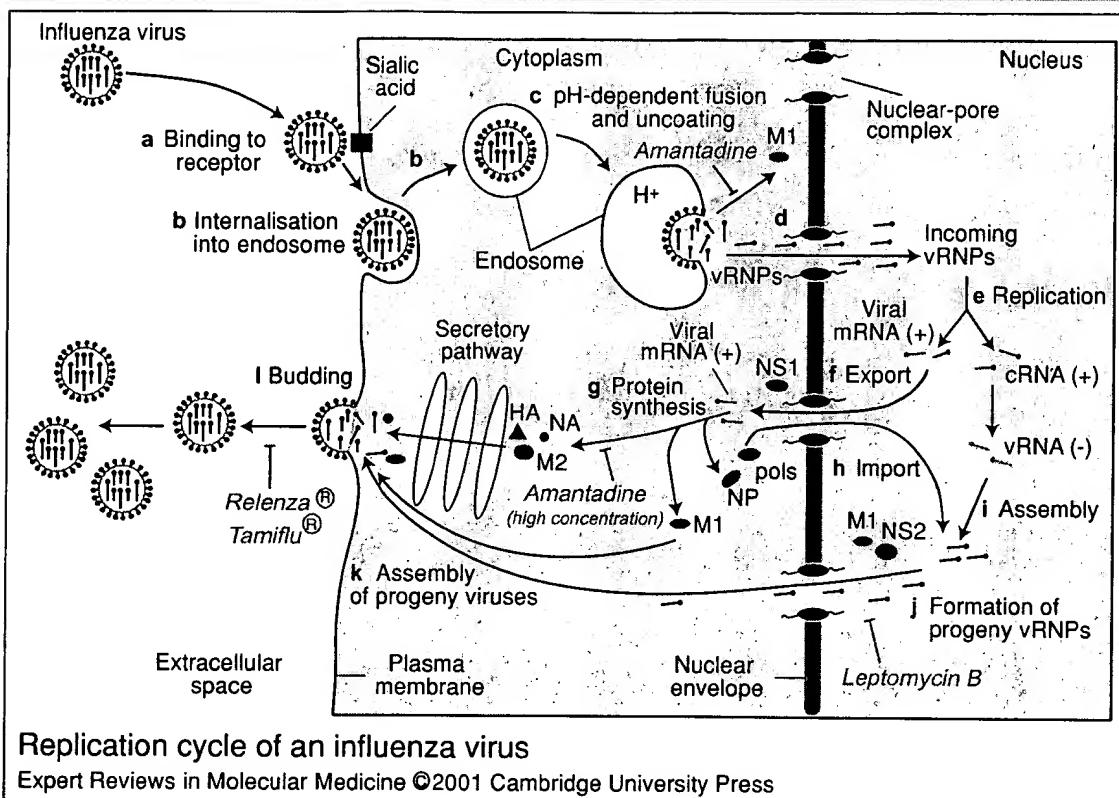


Figure 3. Replication cycle of an influenza virus. (a) The virus binds to receptors on the surface of the host cell and (b) is internalised into endosomes. (c) Fusion and uncoating events, which are pH dependent, result in (d) the release of the viral genome (in the form of viral ribonucleoproteins; vRNPs) into the cytoplasm. The vRNPs are then imported into the nucleus for (e) replication. (f) Positive-sense viral messenger RNAs (mRNAs) are exported out of the nucleus into the cytoplasm for (g) protein synthesis. (h) Some of the proteins are imported into the nucleus to assist in viral RNA replication and (i) vRNP assembly, which also occur in the nucleus. (j) Late in infection, the vRNPs form and leave the nucleus, and (k) progeny viruses assemble and (l) bud from the plasma membrane. The sites of action of anti-viral drugs are shown in red, italic text. Abbreviations used: cRNA (+), positive-sense complementary RNA; HA, haemagglutinin; M1, matrix protein; M2, tetrameric ion channel; mRNA (+), positive-sense messenger RNA; NA, neuraminidase; NP, nucleoprotein; NS1, a non-structural protein, NS2, a viral protein; polys, polymerases; vRNA (-), negative-sense genomic RNA (fig003gwn).

Following the interaction of the virus with a receptor at the cell surface, it is rapidly internalised into clathrin-coated pits – a process that is dependent on dynamin, a cellular GTPase (GTP phosphohydrolase; Ref. 19). Viruses are trafficked through the endocytic pathway and ultimately reach a low-pH compartment (where the pH is approximately 5.5; Refs 20, 21). At this pH, the viral fusion machinery is triggered. HA undergoes a conformational change, forming a ‘coiled-coil’ of α helices and exposing the previously buried hydrophobic fusion peptide, which then inserts into the endosomal membrane (Refs 22, 23). This initiates the fusion

event and releases the interior components of the virus (i.e. M1 and vRNPs) into the cytoplasm.

Uncoating and nuclear import

In addition to triggering fusion, endosome acidification has one other crucial function in virus entry. The presence of the M2 ion channel in the envelope of the virus means that the components inside the virus (i.e. M1 and the vRNPs) become exposed to the low pH of the endosome. Exposure to a low pH is necessary both *in vitro* and *in vivo* to disrupt M1–vRNP interactions and uncoat the virus (Refs 24, 25). Although the acidification of vRNPs is not required for nuclear import per se

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(Ref. 26), it is required to uncoat completely the M1-vRNP complexes, which are otherwise too big to pass through the size-restricted channel of the nuclear-pore complex. The import of vRNPs occurs through nuclear pores and is mediated by nuclear localisation signals on NPs (Refs 27, 28, 29).

It is now known that two closely related anti-viral drugs, namely amantadine (Symmetrel[®]) and rimantadine (Flumadine[®]), target the pH-dependent uncoating event. At micromolar concentrations, amantadine inhibits the nuclear import of vRNPs in cell culture (Ref. 30). The target of amantadine is the M2 ion channel (Refs 31, 32). Amantadine blocks the M2 channel and prevents the acidification of the virus interior. Thus, endosome acidification and virus fusion are not compromised, but M1-vRNP dissociation and ultimately nuclear import are prevented. Amantadine therefore inhibits M2-dependent virus uncoating and is specific for influenza A viruses. At micromolar concentrations, it has no effect on either influenza B or C viruses, which have different ion channels. At higher concentrations (e.g. millimolar), amantadine has additional non-specific effects and can act as a weak base, neutralising the acidic pH in both the endosome and the Golgi apparatus of the cell (Refs 31, 33). As discussed below, the Golgi apparatus affects the HA trafficking of certain influenza viruses through the secretory pathway during the later stages of infection.

Although amantadine and rimantadine are useful for certain at-risk populations, they tend not to be heavily prescribed, mainly owing to the rapid emergence of drug-resistant strains (Ref. 34), and their neurological side effects. Drug resistance is caused by mutations occurring in the nucleotides that encode the amino acids lining the interior of the M2 tetramer. Both amantadine and rimantadine appear to act by the same mechanism, although rimantadine is more commonly used because it has fewer side effects.

Virus replication and transcription

Influenza viruses are one of the few RNA viruses to undergo replication and transcription in the nucleus of their host cells (Ref. 35). In the nucleus, the vRNPs serve as templates for the production of two forms of positive-sense RNA: viral messenger RNA (mRNA) and complementary RNA (cRNA; Ref. 36). The synthesis of mRNA is catalysed by the viral RNA-dependent RNA

polymerase (comprising the three subunits PA, PB1 and PB2), which is part of the incoming vRNP complex. Viral mRNAs are processed in an analogous fashion to other eukaryotic mRNAs; that is, they are capped (i.e. contain a methylated 5' guanosine residue) and are polyadenylated (i.e. contain a sequence of polyadenylic acid at their 3' end), and are exported from the nucleus for translation by cytoplasmic ribosomes. The nuclear export of viral mRNA utilises the 'machinery' of the host cell, but is selective; export is controlled by the viral non-structural protein NS1 (Ref. 37). Many viral proteins (NP, M1, NS2 and the polymerases) are then imported into the nucleus for the final stages of replication and for vRNP assembly. The viral cRNA is neither capped nor polyadenylated, and remains in the nucleus, where it serves as a template for the production of negative-sense genomic RNA (vRNA).

Nuclear export

Following virus replication in the nucleus, the initial trafficking event for virus assembly is the export of the newly formed vRNPs out of the nucleus. This process appears to be a reversal of the nuclear import process because it occurs through nuclear pores (Ref. 35). Nuclear export is blocked by the antibiotic leptomycin B (Refs 38, 39). Leptomycin B binds to the chromosome region maintenance 1 (CRM1) export receptor, the major receptor in protein-based nuclear export pathways in the cell (Ref. 40). Thus, both the nuclear import and export of vRNPs appear to rely on the protein-based host machinery; viral RNA component(s) appear to have no role. Whereas the signals for nuclear import are found on NPs (see Ref. 29 for a review), the signals for nuclear export are less clearly defined. M1 is a major regulator of nuclear transport (Refs 30, 41); however, it might not form part of a stable nuclear export complex and be exported along with the vRNPs because, under certain circumstances, vRNP nuclear export can occur despite the accumulation of M1 in the nucleus (Refs 42, 43). M1 might act in the nucleus during the final stages of vRNP assembly, and the translocation event might depend on nuclear export signals on the NS2 protein (Refs 44, 45), or even on NP itself.

Glycoprotein processing

Role of HA

HA is the best-characterised envelope glycoprotein and is important for the subsequent pathogenesis

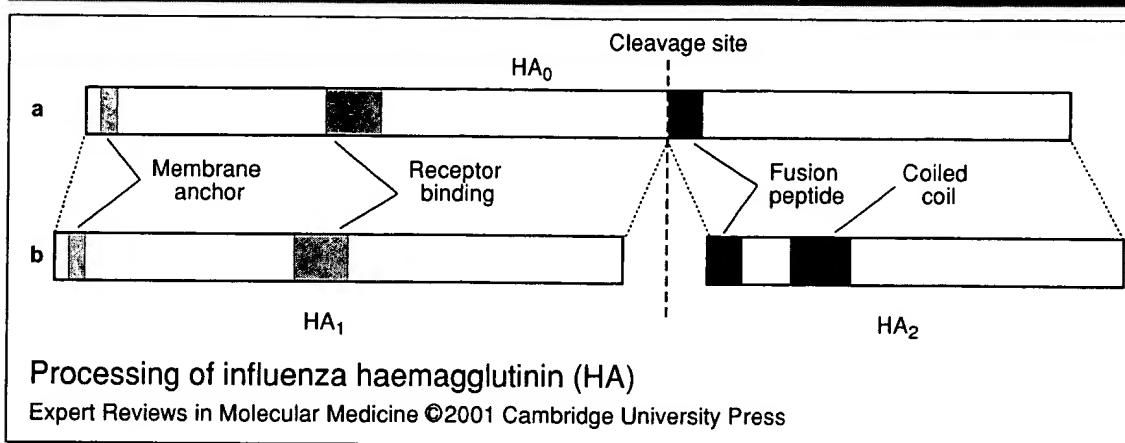


Figure 4. Processing of influenza haemagglutinin (HA). The key regions of HA are shown. (a) The protein is produced as a precursor molecule (HA_0). (b) HA_0 is cleaved by a protease into two active subunits, which are known as HA_1 and HA_2 . HA_1 contains the receptor-binding domain, anchors the protein to the membrane and is held together with HA_2 by disulphide bonds. HA_2 contains the fusion peptide that is activated when a coiled coil of α helices is formed, a process that is dependent on a low pH (fig004gwn).

of the virus. Like all glycoproteins, HA is synthesised in the rough endoplasmic reticulum, and is transported to the cell surface via the Golgi apparatus. HA is synthesised as a precursor molecule (HA_0), which undergoes proteolytic processing into two subunits (HA_1 and HA_2), which are held together by disulphide bonds (Fig. 4). This processing is vital for the subsequent infection of new cells (Refs 46, 47). Without proteolysis, the acid-triggered conformational change in HA to expose the fusion peptide cannot occur, and therefore the virus is essentially non-infectious. The HA cleavage site relies on the presence of basic amino acids. In human influenza viruses, there is a single basic amino acid (arginine; R) at the site of cleavage (e.g. HA_1 -PSIQVR-GL- HA_2). The protease mediating cleavage is thought to be the tryptase Clara, which is released from Clara cells in the epithelial lining of the respiratory tract. The cleavage site is specific and the protease has limited tissue distribution. Both of these features mean that influenza infections are generally limited to the upper respiratory tract. In the laboratory, most cell lines do not support multi-cycle replication unless exogenous trypsin is added. The protease that cleaves HA derives from the host cells, and has not been successfully targeted for therapeutic intervention. By contrast, other viruses, such as human immunodeficiency virus 1 (HIV-1), encode their own proteases, which are excellent targets for anti-viral drugs.

In birds, the situation can be quite different. Whereas most non-virulent or low pathogenicity avian influenza viruses also have a monobasic cleavage site (e.g. HA_1 -PEKQTR-GL- HA_2), highly pathogenic strains have a polybasic cleavage site (e.g. HA_1 -KKREKR-GL- HA_2). Thus, they can be cleaved by ubiquitous proteases, such as furin, which is present in the Golgi apparatus of all cells. These HAs also have a high pH optimum of fusion and the M2 ion channel acts to keep the pH of the Golgi above the threshold for fusion. Following the addition of high concentrations of amantadine to these cells, M2 function is blocked and HA undergoes premature pH-mediated activation, and thus infectious progeny viruses are not produced (Ref. 33). Avian influenza subtypes that have polybasic cleavage sites (e.g. fowl plague virus) are not restricted to particular tissues and can cause fatal systemic infections.

One other factor that enhances pathogenicity is bacterial superinfection (Ref. 46). In humans, influenza viruses are normally confined to the upper respiratory tract; however, influenza viruses can invade a patient's lower respiratory tract if it is colonised by bacteria (e.g. in patients suffering from chronic bronchitis or emphysema). Bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and various Gram-negative bacilli often produce extracellular proteases that can cleave monobasic cleavage sites on HA, enhancing virus spread. In addition,

some bacterial products (e.g. lipopolysaccharide) can activate serum plasminogen, as well as inflammatory host proteases such as kallikrein and factor Xa. These host-derived proteases can cleave certain HAs, facilitating the activation of progeny influenza viruses in the lung and the development of deadly influenza pneumonia.

Complementary role of NA

HA cleavage can clearly be a major factor in influenza virus pathogenesis, but the overall process is complex and is affected by both viral and host factors. Importantly, the 1918 pandemic strain was highly virulent, yet contained an unremarkable monobasic HA cleavage site (Ref. 48). However, the recent H5 'Hong Kong avian flu' that infected humans did contain a polybasic cleavage site (Refs 7, 48). A study of the WSN strain of influenza virus has given rise to some clues about the controlling factors for HA-mediated pathogenesis. WSN is an H1N1 virus that was originally isolated from humans in 1933 and has been adapted to, and is neurovirulent in, mice (Ref. 49). It has been known for many years that WSN has a wider than usual cell tropism in the laboratory, and undergoes multi-cycle growth in other cell types besides the canine cell line MDCK, including Madin-Darby bovine kidney (MDBK) cells (Ref. 50). The NA in WSN viruses has a key C-terminal lysine residue (lys 453) and can bind plasminogen and sequester it close to the infected cell surface. Plasmin, the cleaved, active product of plasminogen, can then cleave and activate HA, allowing influenza virus spread (Ref. 51). However, a C-terminal lysine is present in all influenza N1 strains (including the 1918 strain; Ref. 52), and the functional relevance of plasminogen binding is uncertain outside of the laboratory setting.

Virus assembly at the plasma membrane

For virus assembly at the plasma membrane, it is essential that all of the viral components (i.e. HA, NA, M2, M1 and the vRNPs) are trafficked to the correct physical location in the cell and are correctly processed. M1 is central to this interaction. M1 molecules bind to vRNPs, the plasma membrane (possibly via the cytoplasmic tails of the glycoproteins) and also other M1 molecules to form a shell beneath the virus envelope (Fig. 5b). Originally, M1 was thought to bind to membranes via a large, buried hydrophobic surface (Ref. 53), but has recently

been shown to act as a peripheral membrane protein and to interact via surface electrostatic interactions *in vitro* (Ref. 54). It is possible that the binding of M1 to membranes *in vivo* relies on a combination of both hydrophobic and electrostatic interactions, as well as specific protein interactions with the envelope proteins (Ref. 55). In polarised epithelia, budding of virus particles occurs exclusively from the apical surface (Ref. 56). All of the envelope proteins are localised to the apical surface of polarised cells when expressed individually in heterologous systems, and thus are independently transported to the site of assembly.

Virus budding and release

For the final budding step, it has recently been shown that the viral components coalesce into specific regions of the plasma membrane, which are known as detergent-insoluble glycolipid-enriched domains (DIGs), or lipid rafts. It is likely that these DIGs are specialised sub-compartments of the membrane from which the viruses bud (Refs 57, 58). The formation of viruses at these sites appears to rely on the presence of the cytoplasmic tails of both HA and NA. These glycoproteins, along with M1, M2 and host-cell factors (the actin cytoskeleton and the polarised nature of the cell), appear to control virus morphology, and thus determine the spherical or filamentous nature of the resultant particles (Refs 9, 59, 60). An overall model for virus assembly might involve the initial interaction of M1 with membranes, followed by more-specific interactions of the HA and NA cytoplasmic tails in specialised DIGs.

The final release of viruses from the cell surface relies on the action of the viral NA. NA (sialidase) acts as a receptor-destroying enzyme, by removing sialic acid (the viral receptor) from the surface of host cells (Ref. 61). Without this step, the newly forming virus particles immediately re-bind to their receptor and are not released into the extracellular space. Instead, they remain attached to the cell in large clumps. NA is therefore important for the efficient release of viruses. Indeed, the establishment of a productive infection is dependent on both NA and HA. The carbohydrate residues surrounding the receptor-binding site of HA are known to modulate the affinity of interaction with sialic acid (Ref. 62). Thus, although an increase in the affinity of HA for sialic acid might increase infection, it might counteract the receptor-destroying activity of

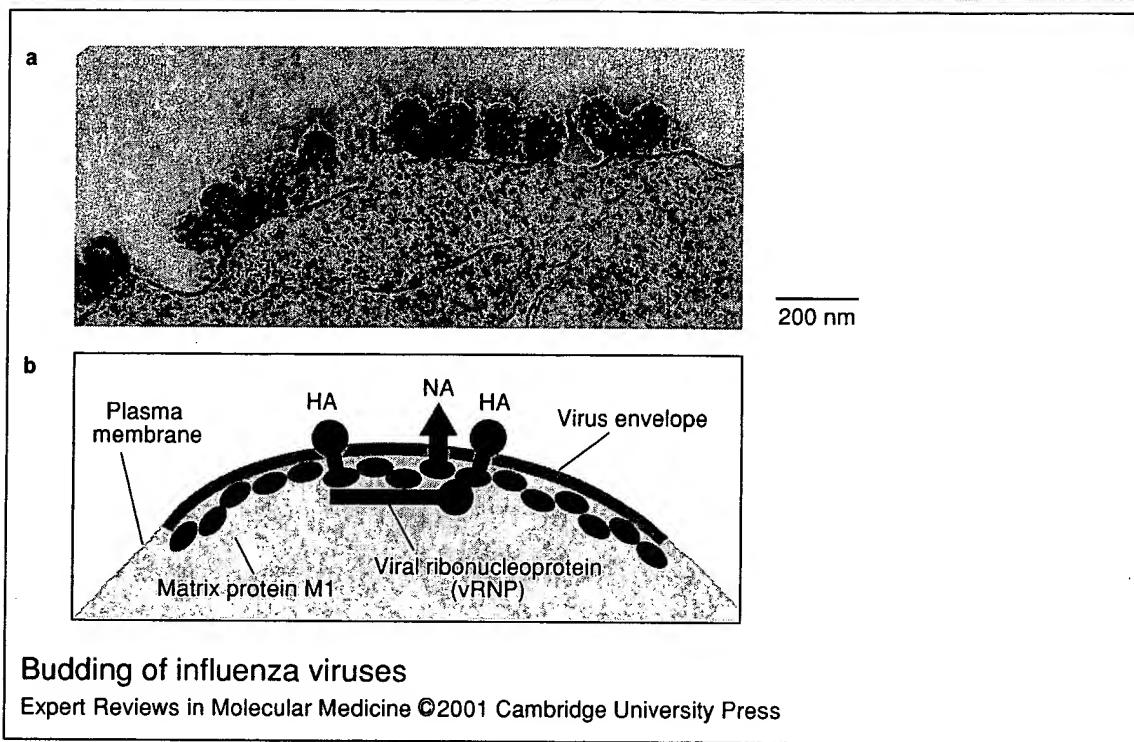


Figure 5. Budding of influenza viruses. (a) This thin-section electron micrograph shows influenza viruses (strain A/WSN/33) budding from the plasma membrane of an infected mouse cell (strain L929). (The micrograph was kindly provided by Melanie Ebersold, Yale University, New Haven, CT, USA). (b) This diagram depicts the molecular interactions that occur when an influenza virus buds from the plasma membrane of a host cell. The matrix protein M1 is central to these interactions. M1 molecules bind to: viral ribonucleoproteins (vRNPs); the plasma membrane, possibly via the cytoplasmic tails of the two surface glycoproteins that are found in influenza A and B viruses, namely haemagglutinin and neuraminidase (HA and NA); and also other M1 molecules to form a shell beneath the virus envelope (fig005gwn).

NA, thereby reducing virus release from the cell surface. Virus release and spread therefore requires a delicate balance between the function of the two glycoproteins.

Recently, the crucial role of NA in the life cycle of the virus has been exploited to great effect for the development of anti-viral drugs. Following the determination of the NA crystal structure (Ref. 63), a concerted effort was made to find small molecules that would bind to, and block, the highly conserved sialic-acid-binding site of NA (Ref. 64). Two analogues of sialic acid, namely Zanamivir (Relenza®) and Oseltamivir (Tamiflu®), have recently been approved for the treatment of influenza (Ref. 65), and other related compounds are currently in development (Ref. 66). These drugs are effective against both influenza A and B viruses. The new inhibitors of influenza NA are significant because they act as anti-viral compounds in a previously unexplored

manner – by preventing virus release and spreading from cell to cell. The development of these drugs is a direct consequence of the information provided by X-ray crystallography; thus, along with HIV-1 protease inhibitors, they can be classed as one of the major successes of rational structure-based drug design.

Conclusions

The molecular events occurring throughout the life cycle of influenza viruses have direct implications for the ability of this potentially deadly virus to cause disease in its host and for new viruses to emerge from animal hosts. From initial virus binding, through pH-dependent internalisation and finally to co-ordinated virus assembly and release, an understanding of the fundamental biology of the virus has yielded information that has allowed both the design of anti-viral drugs as well as an understanding of

the ecology and emergence of the virus. However, many questions still remain regarding the interaction of influenza viruses and their host cells at a molecular level. Although it is well known that sialic acid and a low pH are required for virus entry, it is currently unclear how the virus receptor links to the cellular internalisation machinery for endosomal trafficking. Unlike the case in many other viruses (Ref. 67), the cellular cytoskeleton appears to have only a minor role in the intracellular trafficking of incoming influenza viruses. However, the cytoskeleton might facilitate the transport of the newly formed vRNPs through the cell and the co-ordination of virus assembly at the plasma membrane. Although a great deal of progress has been made on the viral and cellular determinants of overall pathogenicity, a complete understanding of virulence *in vivo* appears to be a long way off. In particular, an intensive effort is under way to find out how the pandemic 1918 strain caused such devastating disease. Thus, it appears that this ever-emerging virus will continue to present new challenges with regard to both our understanding of the basic biology of the virus, and how this relates to the development and application of anti-viral strategies.

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Further reading, resources and contacts

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The Influenza Sequence Database is a curated database of nucleotide and amino acid sequences, produced by the Los Alamos National Library. It is intended to provide the research community with easy sequence deposit and retrieval capabilities, together with tools tailored, in particular, to the analysis of haemagglutinin and neuraminidase sequences. The website also provides many links to other sites, including Genbank, Medline and the Protein Databank (for retrieval of structural records).

<http://www-flu.lanl.gov/>

The Influenza Prevention and Control website, produced by the US Centers for Disease Control and Prevention, Atlanta, GA, is a useful information source.

<http://www.cdc.gov/ncidod/diseases/flu/fluvirus.htm>

All the Virology on the WWW seeks to be the best single site for virology information on the Internet that will be of interest to both the professional virologist and the general public.

<http://www.tulane.edu/~dmsander/garryfavweb.html>

The Virology at Cornell website has been created by the Departments of Plant Pathology and Microbiology and Immunology at Cornell University, NY, USA (produced by Dr Sondra Lazarowitz). It emphasises molecular aspects of virology, focusing on fundamental principles of virus structure, replication, genetics and virus-host interactions that lead to disease development.

<http://ppathw3.cals.cornell.edu/virology/Virology.htm>

Features associated with this article

Figures

- Figure 1. Electron micrograph of an influenza virus (fig001gwn).
- Figure 2. Structure and genome organisation of influenza viruses (fig002gwn).
- Figure 3. Replication cycle of an influenza virus (fig003gwn).
- Figure 4. Processing of influenza haemagglutinin (HA) (fig004gwn).
- Figure 5. Budding of influenza viruses (fig005gwn).

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Intracellular trafficking of influenza virus: clinical implications
for molecular medicine

Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines

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Summary

Background In response to the emergence of severe infection capable of rapid global spread, WHO will issue a pandemic alert. Such alerts are rare; however, on Feb 19, 2003, a pandemic alert was issued in response to human infections caused by an avian H5N1 influenza virus, A/Hong Kong/213/03. H5N1 had been noted once before in human beings in 1997 and killed a third (6/18) of infected people.^{1,2} The 2003 variant seemed to have been transmitted directly from birds to human beings and caused fatal pneumonia in one of two infected individuals. Candidate vaccines were sought, but no avirulent viruses antigenically similar to the pathogen were available, and the isolate killed embryonated chicken eggs. Since traditional strategies of vaccine production were not viable, we sought to produce a candidate reference virus using reverse genetics.

Methods We removed the polybasic aminoacids that are associated with high virulence from the haemagglutinin cleavage site of A/Hong Kong/213/03 using influenza reverse genetics techniques. A reference vaccine virus was then produced on an A/Puerto Rico/8/34 (PR8) backbone on WHO-approved Vero cells. We assessed this reference virus for pathogenicity in in-vivo and in-vitro assays.

Findings A reference vaccine virus was produced in Good Manufacturing Practice (GMP)-grade facilities in less than 4 weeks from the time of virus isolation. This virus proved to be non-pathogenic in chickens and ferrets and was shown to be stable after multiple passages in embryonated chicken eggs.

Interpretation The ability to produce a candidate reference virus in such a short period of time sets a new standard for rapid response to emerging infectious disease threats and clearly shows the usefulness of reverse genetics for influenza vaccine development. The same technologies and procedures are currently being used to create reference vaccine viruses against the 2004 H5N1 viruses circulating in Asia.

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Introduction

In February, 2003, two family members were admitted to intensive care wards in Hong Kong Special Administrative Region with influenza-like respiratory illness. Avian-like H5N1 influenza viruses were isolated from both patients, one of whom succumbed to infection. This was the first time since 1997 that H5N1 viruses had been identified in human beings, and WHO responded by issuing a pandemic alert. Candidate vaccines were immediately sought. The recent outbreak of severe acute respiratory syndrome (SARS) had been a striking example of the rapid and global spread of an emerging infectious disease. However, even the effects of SARS could be dwarfed by those that could arise with the emergence of an influenza pandemic.

Infection caused by the influenza A virus is a zoonosis, and the animal reservoir of this virus is the aquatic bird populations of the world. The compelling epidemiological link between the presence of the virus in poultry in live-bird markets and the appearance of H5N1 in human beings in 1997 suggested that influenza A viruses can be transmitted directly from avian species to man and can cause severe respiratory disease.^{1–3} Although control of the 1997 outbreak was achieved by culling millions of birds in the Hong Kong markets,⁴ this episode demonstrated that the capability for an effective global response to emerging influenza threats was poor because of technical, legislative, and infrastructural limitations. A disturbing finding that emerged from this event was that the scientific community was unable to produce an effective vaccine even after several years.

The inactivated human influenza vaccines in use today are derived from essentially modified viruses. By exploiting the segmented nature of the influenza A genome, vaccine manufacturers and the laboratories of the WHO influenza network have produced a reassortant virus carrying the circulating virus's gene segments that encode haemagglutinin and neuraminidase, the major targets of neutralising antibodies. The remaining six-gene segments are supplied from PR8, a laboratory-adapted avirulent H1N1 strain.⁵ The resulting reassortant virus has the antigenic properties of the circulating strain and the safety and high-yield properties of PR8.

The first batch of inactivated material against the 1997 H5N1 virus was not ready for clinical trial until 7 months after the second case of human infection arose, and even today the effectiveness of vaccine against this virus has not been proven.⁶ A key reason for this delay in the production of an H5N1-specific vaccine was the nature of the virus itself. The H5N1 virus is highly pathogenic in human beings and poultry. The agent must be handled only under conditions of at least biosafety level 3 (BSL3), and it can kill fertilised chicken eggs, the standard medium for the reassortment and

propagation of influenza virus before its inactivation and formulation for use in vaccines. These same traits are present in the 2003 H5N1 virus.

The pathogenic nature of these H5N1 viruses is linked to the presence of additional basic residues in haemagglutinin at the site of cleavage, a step required for haemagglutinin activation and, thus, for virus entry into cells.^{7,9} To overcome the high pathogenicity of the virus, polybasic aminoacids have to be eliminated. A rapid, reproducible system to achieve these modifications—ie, plasmid-based reverse genetics—has been developed only in the past 4–5 years.^{10–12} The potential benefits of reverse genetics for the generation and attenuation of vaccine candidates against highly pathogenic and low pathogenic influenza viruses are enormous.^{13–15} However, the host specificity of the RNA polymerase I promoter used in the influenza reverse-genetics systems and the required use of an approved cell line limits the practical options for the system's use in the manufacture of human vaccines. The vaccine-candidate reference virus stock described in this report has been produced entirely on a cell substrate licensed for the manufacture of human vaccine, and as such, is—to our knowledge—the first reverse genetically derived influenza vaccine suitable for testing in clinical trials. We describe the construction of a vaccine reference virus in Good Manufacturing Practice (GMP)-grade facilities in less than 4 weeks from the time of virus isolation. Our findings highlight the speed with which new technologies can be implemented in response to influenza pandemic alerts.

Methods

Cells and A/Puerto Rico/8/34 plasmids

We obtained WHO-approved Vero cells (WHO-Vero, X38, p134) from the American Type Culture Collection (Manassas, Virginia, USA). Passage-142 cells (five passages since their removal from a working cell bank) were used for the rescue of the vaccine-candidate virus. The plasmids containing the genes from PR8 have been described elsewhere.¹³

Virus propagation, RNA extraction, PCR amplification, and haemagglutinin and neuraminidase gene cloning
We obtained A/Hong Kong/213/03 (H5N1) that had been passaged in eggs from the WHO influenza network. The virus was isolated and propagated in 10-day-old embryonated chicken eggs. Total RNA was extracted from infected allantoic fluid with use of the RNeasy kit (Qiagen, Valencia, CA, USA) in accordance with manufacturer's instructions. Reverse transcription was carried out with the uni12 primer (5'-AGCA AAAGCAGG-3') and AMV reverse transcriptase (Roche, Indiana Biochemicals Indianapolis, USA). The removal of the connecting peptide of the haemagglutinin was done with use of PCR with the following primer sets: (1) Bm-HA-1 (5'-TATTCTCGTCTCAGGGAGCAA AAGCAGGGG-3') and 739ΔR (5'-TAATCGTC TCGTTTCAATTGAGGGCTATTCTGAGCC-3'); and (2) 739ΔF (5'-TAATCGTCTCTGAAA CTAGAGGATTATTGAGCTATAGC-3') and Bm-NS-890r (5'-ATATCGTCTCGTATTAGTAG AAACAAGGGTGT-3'). We amplified the neuraminidase gene of A/Hong Kong/213/03 using the primer pair Ba-NA-1 (5'-TATTGGTCTC AGGGAGCAAAAGCAGGAGT-3') and Ba-NA-1413r (5'-ATATGGTCTCGTATTAGTAGAAACAAAG GAGTTTTT-3'). PCR products were purified and cloned into the vector pHW2000 as described previously.¹¹

Rescue of virus from Vero cells

The rescue of infectious virus from cloned cDNA was done under GMP conditions. Vero cells were grown to 70% confluence in a 75 cm² flask, trypsinised (with trypsin-versene), and resuspended in 10 mL of Opti-MEM I (Invitrogen, Carlsbad CA, USA). To 2 mL of cell suspension we added 20 mL of fresh Opti-MEM I; then, we added 3 mL of this diluted suspension to each well of a six-well tissue culture plate (about 1×10⁶ cells per well). The plates were incubated at 37°C overnight. The next day, 1 µg of each plasmid and 16 µL of TransIT LT-1 transfection reagent (Panvera, Madison, WI, USA) were added to Opti-MEM I to a final volume of 200 µL and the mixture incubated at room temperature for 45 min. After incubation, the medium was removed from one well of the six-well plate, 800 µL of Opti-MEM I added to the transfection mix, and this mixture added dropwise to the cells. 6 h later, the DNA-transfection mixture was replaced by Opti-MEM I. 24 h after transfection, 1 mL of Opti-MEM I that contained 1 µg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemicals, Lakewood, NJ, USA) was added to the cells. About 72 h after the addition of TPCK-trypsin, the culture supernatants were harvested and clarified by low-speed centrifugation; we then injected 100 µL of the clarified supernatant into the allantoic cavity of individual 10-day-old pathogen-free embryonated research grade eggs (Charles River SPAFAS, North Franklin, CT, USA).

Pathogenicity testing in chickens

Ten 4-week-old chickens received intravenous injections of 0·1 mL diluted virus (dilution ratio, 1/10). We monitored chickens for signs of disease for 10 days using the Intravenous Pathogenicity Index, approved by the Office of International Epizootics (OIE). Additionally, we took tracheal and cloacal swabs (in 1 mL of media) 3 days and 5 days after infection, and we did assays for the presence of virus by injection of 0·1 mL into all of three 10-day-old embryonated chicken eggs. Haemagglutination activity in the allantoic fluid of these eggs was assessed after incubation at 35°C for 2 days.

Pathogenicity testing in ferrets

We tested pathogenicity of the vaccine in five young adult male ferrets (Marshall's Farms, North Rose, NY, USA) aged 4–8 months (weight about 1·5 kg) that were shown by haemagglutination inhibition assays to be seronegative for currently circulating human influenza A viruses (H3N2, H1N1) and H5N1 viruses. We anaesthetised the ferrets with inhaled isoflurane, and they were then infected intranasally with 10⁶–50% egg infectious dose (EID₅₀)/mL of vaccine reassortant virus or wildtype virus. We monitored the ferrets once per day for signs of sneezing, inappetence, and inactivity, and we recorded rectal temperatures and bodyweights. 3, 5, and 7 days after infection, the ferrets were anaesthetised with ketamine (25 mg/kg), and we collected nasal washes using 1 mL of sterile phosphate-buffered saline (PBS) containing antibiotics. We measured titres of virus in these washes with EID₅₀ assays.

To further assess the pathogenicity of the viruses, we collected tissue samples from lungs, brain, olfactory bulb, spleen, and intestine for virus isolation and histopathological analysis at the time of death or in the case of three ferrets, after euthanasia at day 3 after infection. The tissues were fixed in 10% neutral buffer formalin, processed and embedded in paraffin, sectioned at 5 µm, stained with haematoxylin and eosin and examined by light microscopy in a blinded fashion.

Stability testing in eggs

To test the stability of the vaccine virus on propagation, we made 16 consecutive passages of the virus in embryonated chicken eggs. A 10^4 dilution of the virus was made in PBS, and 0.1 mL of the solution was injected into the allantoic cavities of all of four 10-day-old embryonated chicken eggs. Eggs were incubated at 35°C for 1.5–2 days. After incubation, each egg was candled to determine embryo viability before chilling at 4°C. We harvested 2 mL of allantoic fluid from each egg harvested, and samples were pooled together, tested for haemagglutination activity, and then reinjected into another four eggs.

Role of the funding source

The sponsor had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report or decision to submit this manuscript for publication.

Results

Alteration of haemagglutinin cleavage site and virus rescue

The first challenge we faced in producing a vaccine against A/Hong Kong/213/03 (H5N1) was to attenuate the virus in preparation for mass production. Previous experiences have shown that removal of the basic aminoacids at the haemagglutinin cleavage site substantially attenuates pathogenic influenza viruses.^{15–17} Using a PCR-based mutagenesis approach, we replaced the cleavage site encoded by the haemagglutinin gene of A/Hong Kong/213/03 (H5N1) with that of the avirulent A/teal/Hong Kong/W312/97 (H6N1) (figure 1); this modified haemagglutinin gene and the neuraminidase gene of A/Hong Kong/213/03 (H5N1) were cloned individually into the vector pHW2000.¹¹ The two resulting plasmids and the six plasmids encoding the remaining proteins of PR8¹³ were transfected into WHO-approved Vero cells under GMP conditions to rescue the vaccine seed virus, Δ213/PR8. 36–48 h after transfection, isolated areas of cytopathic effect could be seen on the Vero monolayers. Although addition of further 1 µg aliquots of TPCK-treated trypsin every 24 h led to a proportional increase in the cytopathic effect, it was not required for successful virus rescue. The candidate vaccine strain grew to high titres on subsequent amplification in eggs (haemagglutination titres of 1024–2048) and did not cause embryo death. The vaccine seed virus was unable to form plaques on Madin-Darby

A/teal/HK/W312/97 (H6N1)



Figure 1: Creation of haemagglutinin protein of candidate vaccine seed

Haemagglutinin protein of the candidate vaccine seed (Δ213/PR8) was produced by replacing the connecting peptide of the A/Hong Kong/213/03 haemagglutinin gene with that of the A/Teal/Hong Kong/W312/97 gene.

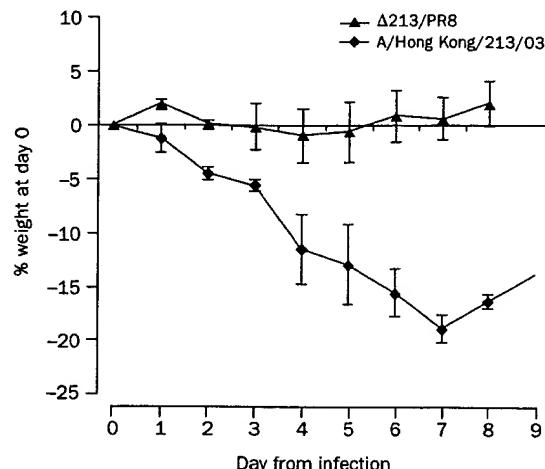


Figure 2: Weight changes of ferrets Infected with wildtype A/Hong Kong/213/03 or Δ213/PR8
Vertical bars show SD.

canine kidney (MDCK) cells in the absence of trypsin, a trait consistent with that of influenza viruses that lack the polybasic cleavage site, and was antigenically indistinguishable from the parental H5N1 virus in haemagglutination inhibition assays. The rescued virus was fully sequenced and was identical to the plasmids used in its creation.

Pathogenicity testing of the candidate reference virus

To assess the pathogenicity of the H5N1 vaccine seed virus, we compared the properties of this virus with those of the wildtype A/Hong Kong/213/03 (H5N1) in ferrets and in chickens. By stark contrast with the wildtype virus, which was lethal to all chickens within 48 h of infection, intravenous administration of a 1/10 dilution of Δ213/PR8 did not result in any signs of infection in chickens, and we were unable to detect any virus in swabs of cloacae or tracheae from inoculated birds. Compared with A/Hong Kong/213/03 (H5N1), Δ213/PR8 was attenuated in ferrets that had been inoculated intranasally with 10^6 EID₅₀ of virus. Ferrets infected with A/Hong Kong/213/03 had inappetence and weight loss (figure 2), with one infected animal dying 6 days after infection and a second killed 10 days after infection because of hind-limb paralysis. Infection in these animals was characterised by viral shedding until 7 days after infection and replication of virus in the lower respiratory tract and olfactory bulb (as determined by virus isolation). In the A/Hong Kong/213/03 infected animals, there was a mild mononuclear cell infiltrate in the meninges and tracheal submucosal mucous glands and an extensive bronchopneumonia. The pneumatic infiltrate progressed in severity from the bronchi to the pleura. The bronchi and bronchioles contained sloughed necrotic epithelial cells, numerous mononuclear cells, and a few neutrophils. The alveoli were consolidated with inflammatory cells and fibrin (figure 3). By contrast, those ferrets infected with Δ213/PR8 did not lose weight (figure 2) and seemed to remain healthy during the study (14 days) (figure 3). Virus was detected in the nasal washes of these animals at 5 days but not 7 days after infection, and virus was recovered from the upper respiratory tract only. By light microscopy, the meninges and trachea of the Δ213/PR8 infected ferrets did not have an inflammatory infiltrate and only a few neutrophils were noted occasionally in pulmonary bronchi. Our results clearly show that

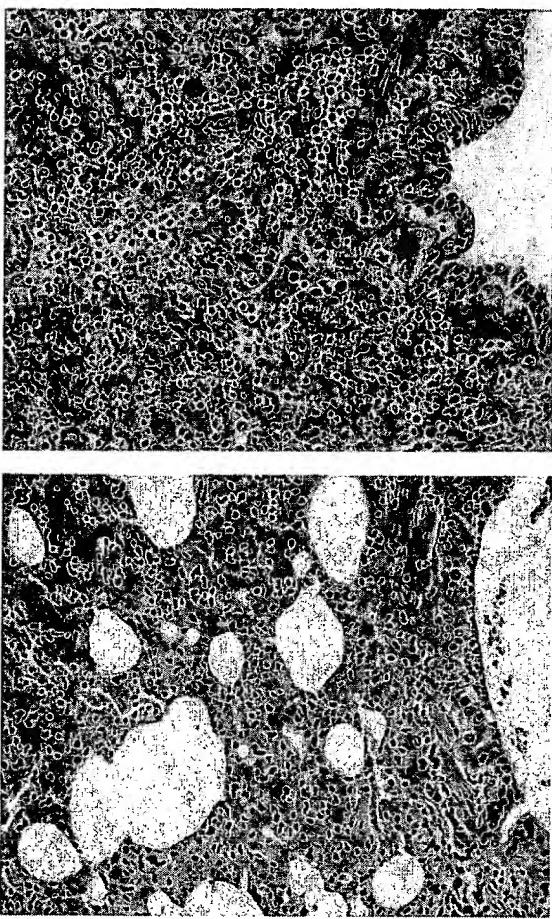


Figure 3: Ferret lung 3 days after infection with wildtype virus (A) and the reverse genetic virus Δ213/PR8 (B)

(A) Alveoli are filled with inflammatory cells and the bronchiolar submucosa is oedematous. (B) Alveoli are free of inflammatory cells and there are a few neutrophils on the surface of the bronchiolar epithelium. Magnification $\times 20$.

$\Delta 213/PR8$ was attenuated. In view of our findings, this virus can be safely handled with standard precautions in BSL2 containment facilities.

Stability of non-pathogenic phenotype

Because the mechanisms and requirements for the accumulation of basic aminoacids at the haemagglutinin cleavage site are not entirely understood, we wanted to confirm that the altered cleavage site remained stable on multiple passages in embryonated chicken eggs. Such passaging in eggs would occur in transition and amplification of the reference virus to vaccine stock. The rescued virus was stable on continued serial passage in embryonated eggs, and we did not detect any change in nucleotide sequence of the haemagglutinin cleavage site after 16 passages. There was no evidence of changing pathogenicity of the virus and we noted only one dead embryo at passage 15. No haemagglutination activity was evident in this egg and no embryo death was seen in passage 16, which strongly suggests that the death was not related to virus replication. Haemagglutination titres at each passage ranged from 512 to 2048 with no apparent trend of increasing or decreasing titres in subsequent passages.

Discussion

The rapid response in terms of potential vaccine reference virus production to the 2003 H5N1 outbreak differs strikingly from the response to the 1997 episode. This difference is attributable to the new scientific technology available in 2003 and, just as importantly, to the infrastructure for virus surveillance in Hong Kong developed since 1997. The first case of H5N1 influenza in Hong Kong was in May, 1997; yet several months elapsed before this virus was finally characterised as an H5N1 virus. In 2003, the causative agent was identified only hours after admission of the patients to the hospital. The increased awareness, surveillance, and availability of reagents to identify influenza viruses of all subtypes bode well for the rapid identification of viruses that arise from future interspecies transfer events and for the coordination of international vaccine development by WHO. The timely distribution of candidate viruses is a very important step in the development of vaccines for pandemic emergencies. Despite the heightened security and documentation requirements for shipping and receiving potential bioterrorism agents, the H5N1 and SARS outbreaks have shown that in true emergencies, global distribution is feasible.

Although it is pertinent to prepare for future pandemics by stockpiling potential vaccine strains, the H5N1 situation in 2003—and the ongoing H5N1 outbreaks throughout Asia in 2004 (<http://www.who.int>)—have highlighted the fact that some of the focus of pandemic planning must go into the implementation of technology to rapidly produce vaccines from field isolates. Although viruses similar to A/Hong Kong/213/03 (H5N1) had been circulating in bird populations, these viruses were antigenically distinct, despite high genetic similarities (Guan Y and Peiris JS, unpublished data). That the aminoacid differences are on the globular head of haemagglutinin and seem to be responsible for much of the antigenic difference means that even a vaccine previously prepared from genetically similar precursor viruses might not provide adequate protection. We may well be faced with potential pandemic situations in the future and the rapid production of a matched vaccine will be needed—a point again highlighted by H5N1 outbreaks in 2004. Although the reference virus described in this report was prepared from a virus isolated in a similar geographic region and only a year earlier, it shares only limited antigenic cross-reactivity to the 2004 H5N1 viruses. Hyperimmune sheep serum samples produced against the purified haemagglutinin of $\Delta 213/PR8$ has at least a six-fold reduced haemagglutination inhibitory activity against A/Vietnam/1203/04 as compared with A/Hong Kong/213/03. As our findings show, we have the technical capabilities to respond rapidly to outbreaks with a safe and stable reference virus, but there is still much to be accomplished before such viruses can be fully used in pandemic and interpandemic influenza vaccine production.

The use of reverse genetics introduces a number of new processes into influenza vaccine manufacture that are not encountered with standard reassortment methods. One of the most obvious is the need for cultured cells. Although both Vero¹⁸ and MDCK^{19,20} cells are in development as substrates for the growth of influenza vaccine, there are additional requirements for the use of cells in reverse genetics. Unfortunately, the number of suitable cell lines is very small. In addition to the regulatory requirements, the choice of cell is also limited by the technology. The plasmid based reverse-genetics systems^{10–12} use the species-specific human RNA polymerase I promoter, which

necessitates the use of cells from primate origin. The Vero cell line is probably the only option currently able to meet both regulatory and technical demands. We have shown that Vero cells can be used to successfully rescue H1N1, H3N2, H6N1, and H9N2 viruses on the PR8 backbone using the 8-plasmid system.²¹ Others have demonstrated the suitability of Vero cells for alternative influenza virus reverse-genetics systems.¹⁰ Although cultures of Vero cells are easily obtained, only cells from fully tested and licensed cell banks are likely to be acceptable for vaccine manufacture. This issue must be acknowledged and access to such cells must be incorporated as part of future pandemic plans.

That future threats of influenza pandemics will be addressed by the use of the technology described in this report seems inevitable. Despite the presence of low pathogenic surrogate strains, the recent human death from influenza-like illness caused by highly pathogenic H7N7 virus in the Netherlands²² reinforces the fact that future outbreaks will probably occur in which this reverse-genetics technology provides the logical—and, possibly, the only—way to respond rapidly and effectively. Although our response to the outbreak of H5N1 influenza in 2003 has shown that current scientific capabilities are sufficient to respond to the threat, there are still legal and infrastructural barriers to be overcome.²³ These barriers include licensing and intellectual property issues surrounding what is, essentially, a genetically modified organism. Yet, these difficulties are not insurmountable and pandemic scares such as the 2003 and ongoing 2004 H5N1 outbreaks are forcing commercial and regulatory parties to address these issues with some urgency. With the development of the 2003 H5N1 vaccine reference virus, and ongoing attempts to create the same for the 2004 virus, the challenge in responding to a threat of an influenza pandemic must now be supported by the large-scale manufacture of the vaccine and by clinical trials of a new vaccine manipulated by reverse genetics.

Contributors

R J Webby, D R Perez, J S Coleman, J H Knight, E I Tuomanen, R G Webster designed the study; R J Webby did much of the construction of the vaccine seed virus; D R Perez developed and constructed plasmid templates; Y Guan and J S Peiris characterised and isolated the initial H5N1 virus; J E Rehg participated in the design and analysis of animal safety testing of the candidate H5N1 vaccine seed virus; E A Govorkova participated in the safety testing of the candidate H5N1 vaccine seed virus; L R McClain-Moss participated in the preparation of GMP documentation of the process and was involved in the reconstitution of the vaccine seed virus.

Conflict of interest statement

None declared. The corresponding author has had full access to all the data in the study and has had the final responsibility for the decision to submit this manuscript for publication.

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Generation of High-Yielding Influenza A Viruses in African Green Monkey Kidney (Vero) Cells by Reverse Genetics

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Influenza A viruses are the cause of annual epidemics of human disease with occasional outbreaks of pandemic proportions. The zoonotic nature of the disease and the vast viral reservoirs in the aquatic birds of the world mean that influenza will not easily be eradicated and that vaccines will continue to be needed. Recent technological advances in reverse genetics methods and limitations of the conventional production of vaccines by using eggs have led to a push to develop cell-based strategies to produce influenza vaccine. Although cell-based systems are being developed, barriers remain that need to be overcome if the potential of these systems is to be fully realized. These barriers include, but are not limited to, potentially poor reproducibility of viral rescue with reverse genetics systems and poor growth kinetics and yields. In this study we present a modified A/Puerto Rico/8/34 (PR8) influenza virus master strain that has improved viral rescue and growth properties in the African green monkey kidney cell line, Vero. The improved properties were mediated by the substitution of the PR8 NS gene for that of a Vero-adapted reassortant virus. The Vero growth kinetics of viruses with H1N1, H3N2, H6N1, and H9N2 hemagglutinin and neuraminidase combinations rescued on the new master strain were significantly enhanced in comparison to those of viruses with the same combinations rescued on the standard PR8 master strain. These improvements pave the way for the reproducible generation of high-yielding human and animal influenza vaccines by reverse genetics methods. Such a means of production has particular relevance to epidemic and pandemic use.

The potential of influenza A viruses to generate new human pathogenic strains from a vast natural reservoir in aquatic birds means that eradication of influenza is not feasible. Correspondingly, disease control requires the monitoring of virus reservoirs and the development of improved antiviral therapies and vaccines. The most widely used human influenza vaccines are those made from subunits of inactivated viruses propagated in embryonated chickens' eggs. These influenza vaccines are essentially genetic modifications of those generated in the mid-1970s, when production was improved by reassorting the circulating strain with A/Puerto Rico/8/34 (PR8), an H1N1 virus adapted for high growth in eggs (49). Since the mid-1970s, the influenza vaccine has been a so-called 6 + 2 reassortant containing the surface hemagglutinin (HA) and neuraminidase (NA) genes from the vaccine target strain and the remaining genes from PR8. Such reassortants are made by coinfecting eggs with both viruses and screening progeny for the desired 6 + 2 configuration.

Although routinely used to prepare human influenza virus vaccines and diagnostic reagents, embryonated chickens' eggs have potentially serious limitations as a host system, not least of which is that the cultivation of influenza viruses in eggs can lead to the selection of variants characterized by antigenic and structural changes in HA (19, 36, 40). Other problems include the lack of reliable year-round supplies of high-quality eggs, the possible presence of adventitious pathogens, and the low

susceptibility of summer eggs to infection with influenza virus (27). The current cycle of interpandemic influenza vaccine production requires detailed planning up to 6 months before vaccine manufacture to ensure an adequate supply of embryonated eggs (10). Because a pandemic event cannot be predicted and a 6-month delay in vaccine production is unacceptable, there is an urgent need to develop improved cell culture systems for vaccine production. Such an improvement is a priority for the World Health Organization (WHO). As part of their Global Agenda on Influenza, the WHO has urged the development of novel vaccines and production strategies or technologies (44).

An additional need for improved cell-based protocols for the production of influenza vaccines has emerged with the development of reverse genetics, which enables the production of influenza vaccines from cloned viral cDNA (7, 16, 30). The ability to custom make influenza viruses by using this technology may dramatically improve the speed with which we can respond to pandemic emergencies. Advantages include the ability to attenuate pathogenic strains (45) and the elimination of the need to screen reassortant viruses for the 6 + 2 configuration, a procedure that can be time-consuming. The potential of reverse genetics to generate vaccine candidates has already been described (15, 39). The main drawback of this methodology is the need to use vaccine-approved cell lines; those commonly used to obtain influenza viruses from cDNA are 293T and Madin-Darby canine kidney (MDCK) cells. The 293T cell line is a transformed cell line and is therefore unlikely to be used for human vaccine production, and there are lingering concerns over the tumorigenic potential of MDCK cells (12). In addition, the use of host-specific RNA polymer-

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ase I promoters in reverse genetics systems limits usable cell lines to those of primate origin.

As an alternative cell-based system, the well-characterized African green monkey kidney (Vero) cell line has potential. Vero cells are suitable for the production of several human virus vaccines, including those against poliomyelitis and rabies (26). Despite earlier findings that influenza viruses do not replicate well in Vero cells (24, 28), the repeated addition of trypsin to the culture medium improves virus yields (20), and preliminary studies with a limited number of strains have indicated that Vero cells support the primary isolation and replication of influenza A viruses (11). Influenza virus vaccines derived from Vero cells have been produced and evaluated for immunogenicity, and their production has been scaled up to commercial levels (2, 23). These Vero-derived vaccines elicit humoral responses comparable to those elicited by the egg-grown vaccines but are more potent stimulators of the cellular response (2). These Vero-based vaccines have, however, consisted of unassorted viruses rather than the accepted 6 + PR8 reassortants.

The aim of the present study was to develop a reproducible, high-yielding Vero-based reverse genetics system to produce influenza vaccine. Govorkova and colleagues (11) previously derived a high-yielding influenza virus by passaging an H1N1 reassortant isolate, A/England/1/53 [HG], several times in Vero cells. This reassortant virus was reported to contain the surface glycoprotein genes from A/England/1/53 and the remaining genes from PR8, the standard vaccine master strain. Our aims were to identify the molecular changes responsible for this high-yielding phenotype and to produce an altered PR8 vaccine master strain adapted for optimal efficiency of viral rescue from cDNA in the eight-plasmid reverse genetics system and for growth in Vero cells.

MATERIALS AND METHODS

Viruses, cells, and plasmids. Influenza viruses were obtained from the repository at St. Jude Children's Research Hospital, Memphis, Tenn., and propagated in 10-day-old embryonated chickens' eggs. MDCK cells and Vero cells approved by the WHO for use in vaccines were obtained from the American Type Culture Collection and maintained in minimal essential medium (MEM; Invitrogen) containing 10% fetal bovine serum. Three variants of A/England/1/53 were used in this study. These viruses were A/England/1/53, which is the wild-type virus, A/England/1/53 [HG], which is a reassortant between A/England/1/53 and PR8 with high growth in eggs, and Vero-adapted A/England/1/53 (Eng53/v-a), which is a Vero-adapted version of A/England/1/53 [HG].

The plasmids carrying the eight gene segments of the high-growth PR8 (H1N1) virus (pHW191 to pHW198) and the HA and NA genes of A/Panama/2007/99 (H3N2) (pHW444 and pHW446, respectively), A/New Caledonia/20/99 (H1N1) (pHW244 and pHW246, respectively), and A/quail/Hong Kong/G1/97 (H9N2) (pHW409 and pHW422, respectively) have been described previously (15). The plasmids carrying the eight gene segments of A/teal/Hong Kong/W312/97 (H6N1) were the same as those used by Hoffmann et al. (16).

Viral RNA extraction, reverse transcriptase PCR, and DNA sequencing. Total RNA was extracted from virus-infected allantoic fluid by using the RNeasy kit (Qiagen). Production of cDNA and PCR were carried out under standard conditions with previously described primers (17). The Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital determined the sequence of template DNA by using synthetic oligonucleotides and rhodamine or dRhodamine dye terminator cycle sequencing ready reaction kits with AmpliTaq DNA polymerase FS (Perkin-Elmer Applied Biosystems Inc., Foster City, Calif.). Samples were subjected to electrophoresis, detection, and analysis on Perkin-Elmer Applied Biosystems model 373, model 373 stretch, or model 377 DNA sequencers.

Viral gene cloning. Full-length cDNA copies of viral genes were amplified by reverse transcriptase PCR as described above. The PCR fragments were cloned

into the vector pCRII-TOPO (Invitrogen) according to the manufacturer's instructions. After transformation of TOP10 cells (Invitrogen) and purification of the plasmid by using a plasmid midi kit (Qiagen), the plasmid was digested with the restriction enzyme BsmBI (New England Biolabs) and ligated into the vector pHW2000 (16). All clones were confirmed by full-length sequencing.

Virus rescue from cloned cDNA. Vero cells were grown to 70% confluence in a 75-cm² flask and then trypsinized with trypsin-EDTA (Invitrogen) and resuspended in 10 ml of Opti-MEM I (Invitrogen). Twenty milliliters of fresh Opti-MEM I was added to 2 ml of cell suspension, and 3 ml of this suspension was seeded into each well of a 6-well tissue culture plate (approximately 10⁶ cells per well). The plates were incubated at 37°C overnight. The following day, 1 µg of each plasmid and 16 µl of TransIT LT-1 (Panvera) transfection reagent were added to Opti-MEM I to a final volume of 200 µl and the mixture was incubated at room temperature for 45 min. After incubation, the medium was removed from one well of the 6-well plate, 800 µl of Opti-MEM I was added to the transfection mix, and this mixture was added dropwise to the cells. Six hours later, the DNA-transfection mixture was replaced by Opti-MEM I. Twenty-four hours after transfection, 1 ml of Opti-MEM I containing L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (0.8 µg/ml) was added to the cells.

The efficiency of virus rescue was calculated by determining the numbers of PFU in the culture supernatants at various intervals after transfection. The number of PFU in MDCK cells was determined as previously described (24).

Viral growth kinetics in Vero cells. The ability of viruses of different genotypes to grow in Vero cells was determined by analyzing multiple replication cycles. Before infection of Vero cells, the rescued viruses were amplified in 10-day-old embryonated chickens' eggs. Confluent Vero cell monolayers grown on 25-cm² plates were washed with phosphate-buffered saline, overlaid with 0.5 ml of diluted virus suspension (to achieve a multiplicity of infection [MOI] of 0.01 PFU/cell), and incubated at room temperature for 60 min. The virus suspension was then removed by aspiration, and 3 ml of MEM containing 0.4 µg of TPCK-trypsin/ml was added. Twenty-four hours later, another 0.4 µg of TPCK-trypsin/ml was added. Sample supernatants were collected at 12-h intervals until 72 h after inoculation. The virus titer of the sample was determined by plaque assay on MDCK cells or by hemagglutination assay with 0.5% chicken erythrocytes. Antigenic analysis was performed with polyclonal antisera against PR8 (H1N1) (goat sera), A/New Caledonia/20/99 (H1N1) (sheep sera), A/Panama/2007/99 (H3N2) (sheep sera), A/teal/Hong Kong/W312/97 (H6N1) (chicken sera), and A/quail/Hong Kong/G1/97 (H9N2) (chicken sera) in hemagglutination inhibition (HI) assays (22). HI assays were performed on three independently rescued virus stocks of each recombinant.

Statistical evaluation of virus replication. The viral growth curves were fitted with logistic function, a nonlinear regression model commonly used for modeling the sigmoid growth curves in biology and chemistry (5, 31, 33). The logistic function in this analysis is given by the following: $\ln(\text{virus titer}) = \phi_1/(1 + \exp[(\phi_2 - t)/\phi_3])$, where t is the time postinoculation (in hours), ϕ_1 is the peak titer (in $\ln[\text{number of PFU per milliliter}]$), ϕ_2 is the half time to peak titer, and ϕ_3 is the time from half to 73% peak titer. The model was fitted using a nonlinear least-square procedure (1) implemented with statistical software S-PLUS (33). Each growth curve was fitted with data from three replicate experiments. Based on the estimates of parameters and their standard errors from fitted models, we compared the parameters for different strains of viruses (PR8, PR8/Eng-NS, and A/teal/Hong Kong/W312/97) by using t tests. Titers of PR8 and PR8/Eng-NS were also compared using the two-way analysis of variance (ANOVA) method separately at 12, 24, 36, and 48 h postinoculation. The two factors of the two-way ANOVA are virus type and master strain backbone.

RESULTS

Viral growth characteristics in Vero cells. Upon multiple passaging of A/England/1/53 [HG] in Vero cells, Govorkova and colleagues (11) produced a high-yielding virus, Eng53/v-a. This reassortant virus was reported to contain the surface glycoprotein genes from A/England/1/53 and the six remaining genes from PR8. To confirm this strain's high-growth phenotype in Vero cells, we evaluated the growth kinetics of the three strains of viruses in Vero cells over 72 h by using hemagglutination activity in culture supernatants as a marker of viral growth. We confirmed the results of Govorkova et al. (11)

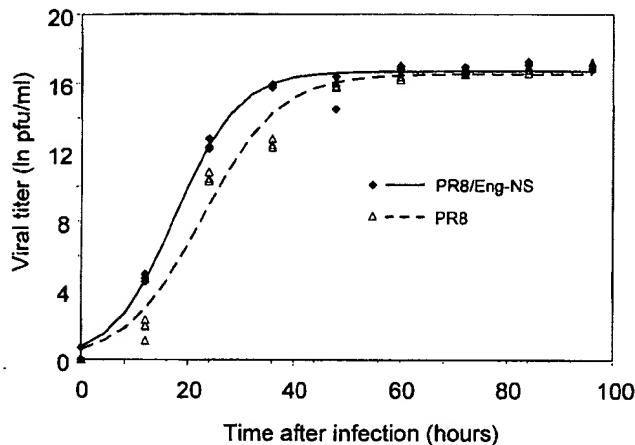


FIG. 1. The efficiency of rescue of PR8 from Vero cells is improved by replacement of the NS gene segment with that from Eng53/v-a. We used the eight-plasmid reverse genetics system to transfect Vero cells and rescue PR8 and PR8 containing the NS gene segment of Eng53/v-a (PR8/Eng-NS). The numbers of PFU in culture supernatants were determined at 12-h intervals. The results shown are from three independent experiments conducted with each virus.

by showing that Eng53/v-a grew faster and to higher titers than did the unpassaged parental A/England/1/53 or PR8.

Genetic characterization of the high-growth phenotype. The Eng53/v-a strain had better growth characteristics in Vero cells than did PR8. To compare the replicative genes of Eng53/v-a and PR8 and thereby identify the molecular differences responsible for the high-growth phenotype, we first sequenced the complete genome of Eng53/v-a. Eng53/v-a contained not only the HA and NA genes from A/England/1/53 but also the nonstructural (NS) gene segment. The genes encoding the remaining Eng53/v-a proteins (PB2, PB1, PA, NP, and M) had more than 99% nucleotide identity to those of PR8. The NS genes from Eng53/v-a had only 90% identity to the corresponding genes in PR8, the HA gene had 98% identity, and the NA gene had 97% identity.

Effect of the NS genes of Eng53/v-a on the efficiency of virus rescue from cDNA in Vero cells. The greatest number of genetic differences between Eng53/v-a and PR8 were in the NS genes. This finding suggested that the NS genes are involved in Vero adaptation. To determine the effect of the NS genes on virus rescue from Vero cells, we cloned the NS gene segment of Eng53/v-a into the plasmid pHW2000 and set up two simultaneous virus rescue experiments. In one we used the plasmids necessary to rescue PR8, and in the other we used plasmids necessary to rescue a reassortant virus containing seven gene segments from PR8 and the NS gene segment of Eng53/v-a (PR8/Eng-NS). Supernatants from each experiment were assayed for virus titers every 12 h. The rescue efficiency of the PR8/Eng-NS virus was superior to that of PR8 12, 24, and 36 h after transfection ($P < 0.001$ [ANOVA]) (Fig. 1), demonstrating that the gene product(s) of the NS genes can influence viral growth characteristics in Vero cells. There was no significant difference between virus titers at times over 36 h posttransfection. The half time to peak titer was significantly ($P < 0.0001$) reduced for PR8/Eng-NS compared to that for PR8 (17.8 ± 0.6 and 22.9 ± 0.7 h, respectively). As a comparison of the virus

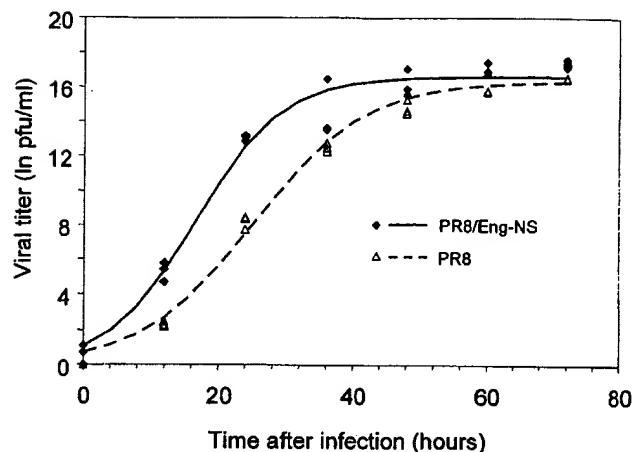


FIG. 2. The growth characteristics of PR8 in Vero cells are improved by inclusion of the NS gene segment of Eng53/v-a. Vero cell monolayers were infected with PR8 or PR8 containing the NS gene segment of Eng53/v-a (PR8/Eng-NS) at a MOI of 0.01. The numbers of PFU in culture supernatants were determined at 12-h intervals. The results shown are from three independent experiments conducted with each virus.

rescue efficiency of PR8/Eng-NS in different culture systems, we also rescued this virus on 293T cells alone and on 293T cells in coculture with MDCK cells. At 24, 48, and 72 h after the addition of trypsin, the titers of virus in the supernatant of the coculture system were comparable to those in Vero (within a fivefold difference), whereas the titers in 293T cells alone were substantially reduced (10^3 - to 10^4 -fold lower). To assess the suitability of the PR8/Eng-NS virus as a master strain for vaccine purposes, we performed experiments in which we rescued the HA and NA of contemporary H1N1 (A/New Caledonia/20/99), H3N2 (A/Panama/2007/99), H6N1 (A/teal/Hong Kong/W312/97), and H9N2 (A/quail/Hong Kong/G1/97) viruses on both the PR8 and PR8/Eng-NS backbones in Vero cells. We were able to rescue all HA and NA combinations on both backbones, although the kinetics of virus rescue (half time to peak titer) were significantly faster with the PR8/Eng-NS viruses ($P < 0.01$ for all virus subtypes).

Effects of the NS genes from Eng53/v-a on growth characteristics of PR8 in Vero cells. To assess the effect of the replacement of the NS gene segment in PR8 on subsequent virus amplification in Vero cells, we infected triplicate samples of near-confluent Vero cells with PR8 and PR8/Eng-NS at a low MOI (0.01) and monitored virus replication by assaying culture supernatants every 12 h. PR8/Eng-NS had a significantly ($P < 0.0001$) lower half time to peak titer than did PR8 (16.8 ± 0.6 and 25.3 ± 0.7 h, respectively), although there was no significant difference in peak titers (1.6×10^7 and 1.2×10^7 PFU/ml, respectively) (Fig. 2). We conducted similar experiments with recombinant PR8 and PR8/Eng-NS viruses carrying the surface glycoproteins of A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), A/teal/Hong Kong/W312/97 (H6N1), and A/quail/Hong Kong/G1/97 (H9N2). The half times to peak virus yield were significantly ($P < 0.0001$) lower in all PR8/Eng-NS viruses than in their PR8 counterparts (Fig. 3). The peak titers of the PR8/Eng-NS viruses carrying the surface glycoproteins of the A/New Caledonia/20/99 and A/Panama/

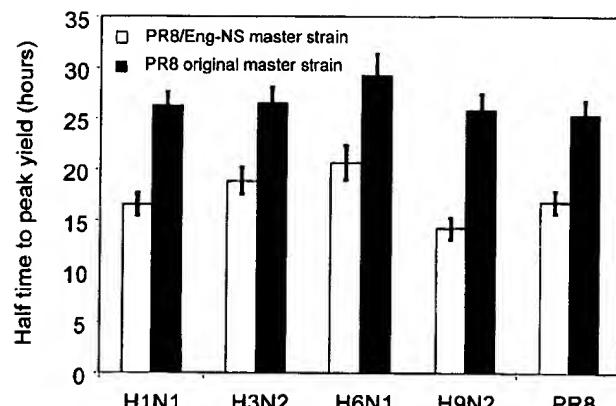


FIG. 3. The half time to peak yield of different subtypes of influenza virus in Vero cells is reduced by inclusion of the NS gene segment of Eng53/v-a. The HA and NA genes of contemporary human H1N1 and H3N2 viruses, contemporary avian H6N1 and H9N2 viruses, and PR8 were rescued on the master strains PR8 and PR8 containing the NS gene segment of Eng53/v-a (PR8/Eng-NS). Vero cell monolayers were infected with each virus at a MOI of 0.01, and the numbers of PFU in culture supernatants were determined at 12-h intervals. The average half times to peak titer shown were calculated from three independent experiments conducted with each virus; the error bars indicate the 95% confidence intervals.

2007/99 viruses were significantly higher ($P < 0.005$) than those of the PR8 viruses (Table 1). The increase in growth kinetics in Vero cells did not affect the ability of the reassortant viruses to grow in eggs. The PR8/Eng-NS variants all grew in eggs to HA titers equivalent to those of their PR8 counterparts (data not shown).

In a related experiment, we compared the growth characteristics, in Vero cells, of two reverse genetics-derived viruses containing the HA and NA genes of A/teal/Hong Kong/W312/97 with those of the reverse genetics-derived wild-type A/teal/Hong Kong/W312/97. The remaining gene segments of the two variants were from PR8 or PR8/Eng-NS. Although the peak titers of all three viruses at 72 h were similar (Fig. 4), the half times to peak titer were significantly ($P < 0.0001$) shorter for the PR8 and PR8/Eng-NS variants (29.3 ± 1.0 and 20.7 ± 0.8 h, respectively) than for A/teal/Hong Kong/W312/97 (60.3 ± 4.5 h) (Fig. 4).

To test whether changes in genes other than NS could lead

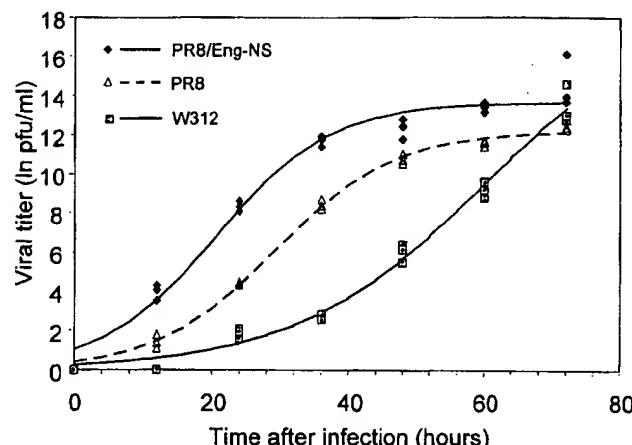


FIG. 4. Growth kinetics of an H6N1 virus are significantly improved by substituting all but the HA and NA genes of the H6N1 virus with genes from a master strain (PR8 or PR8/Eng-NS). We determined the growth kinetics, in Vero cells, of A/teal/Hong Kong/W312/97 (W312) and of reassortant viruses carrying the HA and NA genes of A/teal/Hong Kong/W312/97 and the remaining genes of the PR8 or PR8/Eng-NS master strain. Vero cell monolayers were infected with each virus at a MOI of 0.01, and the numbers of PFU in culture supernatants were determined at 12-h intervals.

to further improvements in the growth characteristics of PR8/Eng-NS, we determined the growth characteristics of PR8, Eng53/v-a, and PR8/Eng-NS in Vero cells. If further improvements were possible, they would be indicated by Eng53/v-a's having growth characteristics superior to those of PR8/Eng-NS. The growth characteristics of Eng53/v-a and PR8/Eng-NS were substantially better than those of PR8 but indistinguishable from each other (Fig. 5). This result suggests that the high-yielding phenotype of Eng53/v-a is due solely to the NS genes.

Stability of viruses rescued from Vero cells. Vaccine production procedures must allow the virus produced to retain the antigenic properties of the parent viruses. Any benefit from an increase in yield would be negated by changes in the antigenicity of a vaccine. To determine whether antigenic changes occurred upon rescue of viruses in Vero cells, we used HI assays to compare the antigenic profiles of the rescued PR8/Eng-NS viruses with those of their wild-type counterparts. HI

TABLE 1. Estimated peak titers of viruses with HA and NA combinations rescued on different vaccine backbones in Vero cells

Source of HA and NA	Backbone	Estimated peak titer ^a	95% Confidence interval	P value
PR8	PR8/Eng-NS	16.59	16.15–17.03	0.4854
	PR8	16.33	15.76–16.90	
A/New Caledonia/20/99	PR8/Eng-NS	16.87	16.44–17.30	0.0041 ^b
	PR8	15.81	15.27–16.34	
A/Panama/2007/99	PR8/Eng-NS	16.48	15.99–16.96	0.0001 ^b
	PR8	14.90	14.34–15.46	
A/quail/Hong Kong/G1/97	PR8/Eng-NS	16.29	15.88–16.70	0.2589
	PR8	15.87	15.28–16.46	
A/teal/Hong Kong/W312/97	PR8/Eng-NS	13.66	13.18–14.14	0.0011 ^b
	PR8	12.18	11.59–12.78	

^a Expressed as natural log of number of PFU per milliliter.

^b Indicates subtypes for which the peak titer of the PR8/Eng-NS variant was statistically higher than that of the PR8 variant.

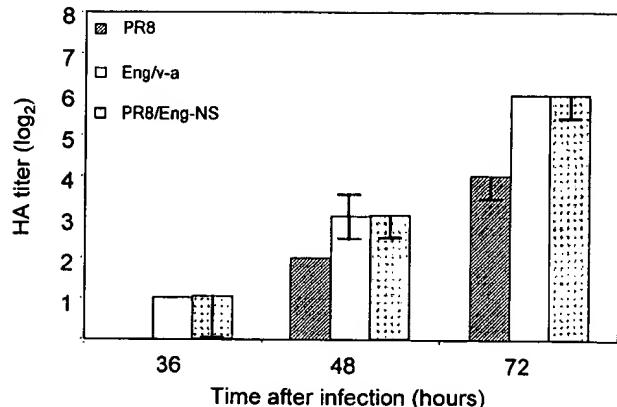


FIG. 5. The NS gene segment of Eng53/v-a is alone sufficient to confer a high-growth phenotype on PR8. Vero cell monolayers were infected with PR8, Eng53/v-a, or PR8 containing the NS gene segment of Eng53/v-a (PR8/Eng-NS) at a MOI of 0.01. Virus titers in culture supernatants were estimated by using hemagglutination assays at 12-h intervals. The data represent median titers from three independent experiments, and the error bars indicate the range of values.

assays were performed using polyclonal antisera against the corresponding wild-type virus. All PR8/Eng-NS rescued viruses were antigenically indistinguishable from the corresponding wild-type viruses. To confirm the genetic stability of the PR8/Eng-NS rescued viruses, we compared the HA gene sequences of the rescued and wild-type viruses. We found no changes in any of the viruses. This result demonstrates that introduction of the NS gene segment from Eng53/v-a into the PR8 vaccine master strain does not lead to changes in the HA gene.

DISCUSSION

Recent discoveries in influenza pathogenicity and reverse genetics have the potential to revolutionize the way we prepare and manufacture pandemic and interpandemic influenza vaccines. Much of this technology has, however, been confined to experimental protocols; the realization of this potential awaits refinements of the methods. The results we report here go a long way toward helping to fulfill the potential of these technologies. By incorporating the NS gene segment of Eng53/v-a into the standard PR8 vaccine master strain, we have shown a reproducible improvement in vaccine virus rescue and growth in Vero cells with no drop in virus titers in eggs. Although we were able to rescue these viruses with the standard PR8 vaccine strain, the improved efficiency of rescue with our system may be crucial with other HA and NA combinations that are poorly infective in Vero cells.

With each of the HA and NA subtypes we tested, the PR8/Eng-NS viruses reached peak titers significantly faster than did the corresponding PR8 virus (Fig. 3). The peak titers of the PR8/Eng-NS variants containing the surface glycoproteins of the two contemporary vaccine strains (A/New Caledonia/20/99 and A/Panama/2007/99) and of A/teal/Hong Kong/W312/97, a virus implicated in the genesis of the 1997 H5N1 human viruses (18), were also significantly higher than those of the PR8 viruses (Table 1). In contrast, there was no difference in the

peak titers of the PR8/Eng-NS and PR8 viruses carrying the surface glycoproteins of A/quail/Hong Kong/G1/97 or PR8 itself. However, by the time these peak titers were reached with the PR8 variants, the cells infected with the PR8/Eng-NS variants had been completely destroyed by cytopathic effects, and a manufacturing process in which cells and fresh media can be continually added or replenished is likely to produce higher yields for those viruses on the PR8/Eng-NS backbone.

Our data also provide support for the continued use of 6 + 2 high-growth reassortants for vaccine production in Vero cells. For example, the PR8/Eng-NS variant of the H6N1 virus had growth characteristics significantly superior to those of the wild-type H6N1 virus; half times to peak titers were three times longer in the wild-type virus. The use of 6 + 2 reassortants also reduces the risks of growing adventitious agents with influenza viruses isolated directly from clinical samples.

One of the benefits of cell-based production of vaccines is that it appears to allow the amino acid sequences of influenza virus HA molecules to remain unchanged; these molecules are altered during the adaptation of viruses to eggs (19, 36, 40). It should, however, be noted that changes in the biologic activity of a virus can occur in the absence of sequence changes. It has been shown that differences in the abilities of the same human H3N2 strain to agglutinate human and chicken red blood cells were associated with the type of cells from which the strain was isolated (MDCK or Vero) (13). Romanova and colleagues have recently shown that the inability of the Vero-grown variant to grow in eggs and agglutinate chicken red blood cells is related to the higher proportion of oligosaccharides of high mannose type in this variant than in the corresponding MDCK-derived isolates. This observation was made in the absence of any amino acid differences between the variants (37). We found that the HA genes of the PR8/Eng-NS-derived viruses were the same before and after rescue and propagation from Vero cells. This stability is an advantage in a vaccine that derives much of its protective qualities from the production of neutralizing antibody directed against the HA molecule. However, as discussed by Kemble and Greenberg (21), such benefits will be lost unless candidate vaccine viruses are first isolated in approved cell lines rather than in the widely used MDCK cells or eggs. The retention of these benefits of cell-based vaccine production requires a commitment from many agencies, including the WHO influenza network.

Host range in influenza viruses is a polygenic trait for which many influenza virus proteins have been implicated (14, 41, 43, 46). Likewise, many genes, including the NS genes, have been implicated in the attenuation of influenza viruses in different hosts (4, 25, 43). However, in our system, the transfer of the NS gene segment from Eng53/v-a to PR8 was alone sufficient to confer the high-growth phenotype (Fig. 5). Although many factors have been reported to determine the efficiency of growth of influenza viruses, the NS1 protein (one of the two proteins expressed by the NS gene segment) is thought to play a significant role in translation and replication. For example, NS1 and its interaction with host proteins have been reported to play central roles in inhibiting the nuclear export and splicing of host mRNA (8, 29, 34, 35, 47, 48). Furthermore, NS1 protein plays an important role in regulating interferon activity (42). It is unlikely, however, that the interferon pathway is involved in viral growth in Vero cells, because Vero cells do

not produce interferon (6). Another study failed to identify major changes in the shutoff of host protein synthesis or in viral protein expression in Vero cells after infection with NS1 deletion mutant viruses (38). Garcia-Sastre et al. showed, however, that a mutant virus that did not express NS1 protein (delNS1) grew approximately 10 times slower on Vero cells than did the wild-type strain (9). The second protein encoded by the NS gene segment is the NS2 protein, or nuclear export protein (NEP). Although little is known about the function of NEP, this polypeptide interacts with nucleoprotein and contributes to the nuclear export of the viral ribonucleoproteins (32). Additionally, amino acid residues of the NEP have been shown to be crucial for viral replication (3). Studies are ongoing to identify the NS gene products responsible for increased viral replication in Vero cells and the mechanisms involved.

The applicability of reverse genetics to the production of influenza vaccines depends on the use of a suitable cell culture system. Technical constraints and the limited number of cells licensed for vaccine production severely limit the options available. It is therefore likely that timely improvements in reverse genetics-derived vaccines will arise from optimization of current protocols rather than identification of alternative systems. The data presented in this paper show that use of the Vero cell system with an improved master strain virus is a viable option for the rapid manufacture of influenza vaccines in pandemic emergencies and for the production of vaccines during annual epidemics. Although the technologies are now available, use of the vaccines created by them requires approval by regulatory agencies, which must be prepared and equipped to rapidly give such approval.

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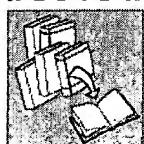
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REVIEW



Generation of influenza A virus from cloned cDNAs — historical perspective and outlook for the new millennium

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SUMMARY

Influenza virus reverse genetics has reached a level of sophistication where one can confidently generate virus entirely from cloned DNAs. The new systems makes it feasible to study the molecular mechanisms of virus replication and pathogenicity, as well as to generate attenuated live virus vaccines, gene delivery vehicles, and possibly other RNA viruses from cloned cDNAs. During the next decade, one can anticipate the translation of influenza virus reverse genetics into biomedically relevant advances. Copyright © 2002 John Wiley & Sons, Ltd.

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INTRODUCTION

The large group of negative-sense RNA viruses comprises the families *Paramyxo*-, *Rhabdo*-, *Filo*-, *Orthomyxo*-, *Arena*- and *Bunyaviridae*, which include numerous human pathogens, such as influenza (*Orthomyxoviridae*), measles (*Paramyxoviridae*) and Ebola virus (*Filoviridae*), and therefore have considerable medical and economical importance. Negative-sense RNA viruses share an RNA genome of negative-polarity, that is, the viral RNA (vRNA) is complementary to the mRNA. Thus, in contrast to positive-sense RNA viruses, the naked RNA genome is not infectious. The negative-sense RNA is assembled with the nucleoprotein and polymerase proteins into viral ribonucleoprotein (vRNP) complexes, which constitute the template for the replication and transcription of the viral genome.

The generation of negative-sense RNA viruses from cloned cDNA is called reverse genetics, a process requiring functional RNP complexes. This

task has been accomplished for nonsegmented negative-sense RNA viruses of the *Rhabdo*-, *Paramyxo*- and *Filoviridae* families. In 1994, Schnell *et al.* [1] reported the generation of infectious rabies virus, a rhabdovirus, from cloned cDNA. Plasmids encoding the full-length viral genome, as well as the nucleoprotein and polymerase proteins (all under the control of the T7 RNA polymerase promoter) were transfected into cells that had been infected with recombinant vaccinia virus expressing T7 RNA polymerase. T7 RNA polymerase transcribed full-length rabies RNA, which was then replicated and transcribed by the polymerase and nucleoproteins, thus initiating the viral replication cycle. Since then, similar systems have been described for a numerous viruses representing the above three families of nonsegmented negative-sense RNA viruses [2–19].

The genomes of segmented negative-sense RNA viruses had long been refractory to reconstitution from cloned cDNAs, likely because of the technical difficulties of providing several vRNPs, together with the nucleoprotein and polymerase proteins. In 1996, Bridgen *et al.* [20] described the generation of Bunyamwera virus (family *Bunyaviridae*, whose genome comprises three segments) from plasmids, marking the first generation of a segmented negative-sense RNA virus from cloned cDNA. The production of influenza A viruses was

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Abbreviations used

CAT, chloramphenicol-acetyltransferase; GP, glycoproteins; HA, haemagglutinin; IFN, interferon; IRES, internal ribosomal entry site; IRF-3, interferon regulatory factor 3; NA, neuraminidase; NES, nuclear localisation signal; NP, nucleoprotein; PKR, protein kinase; RNP, ribonucleoprotein; vRNP, viral ribonucleoprotein.

even more challenging, since their genome consists of eight segments of RNA. Here, we review events in the course of reverse genetics evolution, from early attempts to reconstitute influenza RNP complexes, to the development of systems for introducing mutations into the influenza virus genome, and ultimately to the generation of influenza A virus from cloned cDNAs.

INFLUENZA VIRUS REPLICATION

The three largest RNA segments of the influenza virus genome encode the polymerase proteins PB2, PB1 and PA [21, reviewed in 22]. The nucleoprotein (NP) that encapsidates the RNA is encoded by the fifth segment, while the fourth and sixth segments encode the viral glycoproteins haemagglutinin (HA) and neuraminidase (NA). The seventh and eighth segments both have the coding capacity for two proteins. The viral matrix protein (M1) is translated from a colinear mRNA derived from the seventh segment, whereas the ion channel protein (M2) is encoded by a spliced mRNA from the same segment. A colinear mRNA from the eighth segment gives rise to the nonstructural protein NS1. NS2, by contrast, is the product of a splicing event from the same pre-mRNA.

The influenza virus life cycle starts with the binding of HA to sialic acid-containing glycoconjugates on the cell surface, followed by receptor-mediated endocytosis [reviewed in 22]. The low pH in late endosomes triggers a conformational change in the HA that leads to fusion of the viral and endosomal membranes and the release of vRNPs into the cytoplasm. vRNPs are then imported into the nucleus where replication and transcription take place. The vRNA serves as a template for both the synthesis of a capped and polyadenylated mRNA, as well as a full-length antigenomic complementary RNA (cRNA), which in turn serves as a template for vRNA synthesis. Newly synthesised NP and polymerase proteins are imported into the nucleus and assembled with vRNA to form vRNPs. Late in infection, newly synthesised vRNPs are exported from the nucleus to the cytoplasm, mediated by the NS2 and M1 proteins. At the plasma membrane, vRNPs are assembled into virions that are released subsequently. Thus, in order to generate influenza A virus from cloned cDNAs, all eight viral RNAs

have to be provided in the cell nucleus, together with the three polymerase proteins and the NP.

RECONSTITUTION OF FUNCTIONAL RNP COMPLEXES

The first step towards the generation of influenza virus was the reconstitution of functional RNP complexes. Such complexes can be isolated from detergent-treated virus [23], or assembled from purified NP and polymerase proteins [24], or from proteins expressed in insect cells [25]. Short synthetic RNAs [26,25] or full-length vRNAs [27] were transcribed by the reconstituted RNP complexes, proving the functional integrity of the polymerase complex. Because of the inherent technical difficulties, the purification of viral proteins and their reassembly with synthetic RNAs to form functional vRNP complexes was not a feasible approach to the artificial generation of influenza viruses entirely from cloned cDNAs. Nevertheless, these early studies set the stage for the subsequent advances by demonstrating that the three polymerase proteins and NP are sufficient for transcription and replication of viral RNAs.

SYSTEMS FOR STUDY OF INFLUENZA VIRUS REPLICATION AND TRANSCRIPTION

After demonstrating that functional RNPs can be reconstituted from the polymerase and NP proteins, researchers established cell culture systems to study influenza virus replication and transcription. The three polymerase proteins and the NP were expressed from vaccinia virus [28] or SV40 [29] expression systems, or from cell lines stably expressing all four viral proteins [30]. In these systems, RNA was transcribed *in vitro* and mixed with purified polymerase and NP proteins to form vRNPs, which then were transfected into cells expressing the polymerase and NP proteins. Transfection of naked vRNA into SV40 or vaccinia virus-infected cells expressing the polymerase subunits and NP also resulted in replication and transcription of the vRNA [29,31]. Thus, naked influenza virus RNA is recognised by the polymerase and NP proteins, encapsidated and amplified. These cell culture systems allowed the noncoding regions of vRNAs to be modified, permitting identification of viral promoter elements and regulatory signals. They also allowed mutagenesis of the polymerase and NP proteins,

enabling study of their functions in replication and transcription, as exemplified by a recent study of Perales *et al.* [32]. A potential disadvantage of these systems is the fact that the ratios of the polymerase to NP proteins may not be the same as in virus-infected cells, which may affect viral transcription and/or replication. Nonetheless, this research showed that replication and transcription of artificially generated vRNPs can be achieved in cells.

HELPER VIRUS-BASED GENERATION OF INFLUENZA VIRUS

Armed with the knowledge that functional vRNPs can be reconstituted and introduced into cells, researchers began to contemplate strategies to tackle the next step in the generation of influenza virus — the introduction of artificially generated vRNPs into virions. After transcription of wild-type or mutated cDNA, the resultant vRNA has to be assembled with polymerase and NP proteins to reconstitute vRNPs, which are then mixed with the remaining viral RNPs for the generation of infectious virus. Several strategies, described below, were developed to achieve this goal.

Ribonucleoprotein transfection method

Luytjes *et al.* [33] were the first to establish a system for the generation of influenza that contained vRNA derived from cloned cDNA. They replaced the coding region for the NS gene with that of the reporter gene chloramphenicol-acetyltransferase (CAT). The 5' and 3' noncoding regions containing the influenza virus promoter elements remained unaltered. This NS-CAT-NS cassette was flanked by T7 RNA polymerase promoter sequences and a recognition site for a restriction endonuclease to generate the 3' end of the transcript. The resultant plasmid was transcribed *in vitro* and mixed with polymerase and NP proteins (purified from virions) to reconstitute RNP complexes. The *in vitro* assembled vRNPs were transfected into eukaryotic cells before or after infection with helper influenza virus to provide the remaining vRNPs. CAT expression in lysates from vRNP-transfected and virus-infected cells demonstrated that a population of newly generated virus contained the NS-CAT-NS vRNA in addition to the requisite eight viral RNAs [33]. However, CAT activity was greatly reduced after three passages in cells, indicating

that the CAT segment was not stably maintained [33]. Enami *et al.* [34] were the first to replace a viral gene segment with its respective mutated counterpart (Figure 1; see section on neuraminidase gene for details). This feat required selection of a mutated virus against the background of wild-type helper virus (described in sections on neuraminidase, haemagglutinin M and NP, NS and PB2 genes). Subsequent reports described modifications or improvements of the basic RNP transfection method [35–38].

A strategy similar to the one outlined by Luytjes *et al.* [33] was reported by Seong and Brownlee [39]. Instead of using polymerase and NP proteins purified by gradient centrifugation, these investigators treated isolated RNPs with micrococcal nuclease to remove the RNA and assembled the resulting RNP complexes into vRNPs, using *in vitro* synthesised vRNA.

RNA polymerase I method

A different route was explored by Neumann *et al.* [40], who established the RNA polymerase I system for the alteration of influenza virus genes. In contrast to the RNP transfection method, the RNA polymerase I system does not require *in vitro* RNA transcription, protein purification, *in vitro* RNP reconstitution and transfection of RNP complexes. Rather, a cDNA encoding CAT was inserted between the 5' and 3' noncoding

In vitro transcription

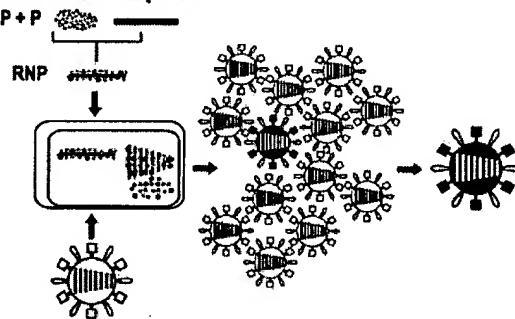


Figure 1. RNP transfection method for the modification of influenza virus genes. To provide reconstituted vRNPs, *in vitro* synthesised vRNA is mixed with purified NP and polymerase (P) proteins, and the resulting vRNP complex is transfected into eukaryotic cells. The remaining seven vRNPs are provided by helper virus infection. Selection systems based on temperature-sensitivity, host-range restriction, drug-sensitivity or antibody-selection are then used to select the recombinant virus from a large pool of viruses composed mainly of helper virus

regions of an influenza cDNA, and this cassette was fused in negative orientation to RNA polymerase I promoter and terminator sequences. RNA polymerase I transcribes ribosomal RNA (rRNA), which, like influenza vRNA, does not contain 5' cap and 3' polyA structures. RNA polymerase I also localises to the nucleus, the site of influenza virus replication. Thus, transfection of a recombinant plasmid encoding an influenza virus-like CAT RNA flanked by RNA polymerase I promoter and terminator sequences leads to CAT vRNA synthesis by cellular RNA polymerase I. As in the methods described above, cells were infected with influenza virus to provide the remaining vRNPs.

Advances with helper virus-dependent reverse genetics

The systems outlined above allowed the generation of influenza virus containing either mutations in one of the viral gene segments or an additional gene segment. Thus, it was possible to undertake extensive mutagenesis of the influenza virus promoter sequences and the putative polyadenylation signal, to elucidate the functions of viral proteins and their role in the viral life cycle, and to address the stability of additional gene segments.

Influenza virus promoter

The twelve 3' terminal and thirteen 5' terminal nucleotides of the eight viral RNA segments of influenza A virus are partly complementary and highly conserved among themselves, as well as among all strains of the virus. Hence, they were thought to constitute the viral promoter. The methods discussed above were exploited to conduct extensive mutagenesis of every nucleotide within these conserved terminal regions. The studies revealed that 12–14 nt at the 3' end of the vRNA and 11–13 nt at 3' end of the cRNA are sufficient for replication and transcription, and therefore constitute the core promoter [26,36,41] [39,42–49]. Further mutagenesis and polymerase protein binding studies demonstrated that both the 3' and 5' ends of the vRNA or cRNA act in concert to constitute the vRNA or cRNA promoter [44,50,51]. Thus, the promoter activity of constructs containing mutations at the 3' end of the vRNA can be restored by introducing

compensating mutations at the 5' end of the vRNA, or vice versa [44,46–48].

Two categories of mutations were found within the core promoter region: those detrimental to promoter activity and those that could be compensated for by replacements at the other end of the respective RNA. The data emerging from mutagenesis experiments indicated that the promoter consists of two regions [45,48]: region I (nucleotides 1–9 at the 3' end of the RNA and nucleotides 1–9 at the 5' end of the respective RNA), in which specific nucleotides are crucial for promoter activity [45,48], and region II (nucleotides 10–15 and 11–16 at the 3' and 5' ends of the vRNA), wherein base pairing is essential [44,46,48,49]. In the latter, the promoter activity is abrogated by mutations that destroy a base-pair, but can be restored by the introduction of mutations that restore base pairing. The two regions are likely connected by a flexible joint formed by an unpaired nucleotide at position 10 at the 5' end of the vRNA [46,48].

On the strength of these findings, several structural models for the influenza virus promoter were proposed. The 'panhandle' model [52] (Figure 2A) predicts a partly complementary double-stranded region encompassing nucleotides 1–16 of the 5' and 3' ends of the vRNA or cRNA. Although based on nuclease S1 protection assays and electron-microscopy studies, the model lacks direct experimental evidence. The 'fork' model [44,45,49] (Figure 2B) proposes a single-stranded conformation for region I and a double-stranded element for region II. The 'corkscrew' model [48,53] (Figure 2C) differs from the other models by predicting short base-paired regions within the 5' and 3' ends of region I, rather than between them: base pairs formed between the nucleotides at positions 2 and 9, and 3 and 8, at both ends of the vRNA stabilise exposed single-stranded RNA structures formed by nucleotides at positions 4–7. Findings by Pritlove *et al.* [54] (Figure 2D) suggest a functional stem-loop structure at the 5' end but not at the 3' end of the vRNA.

The majority of promoter studies were performed with influenza A virus; however, a transcription assay has also been established for influenza B virus [55]. Both viruses share similar features in their promoter structures. The influenza B virus promoter, like its influenza A virus counterpart, consists of two regions; region I, in

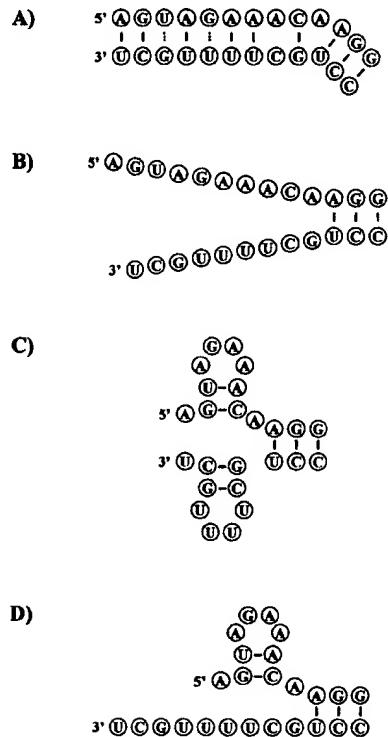


Figure 2. Influenza virus promoter models. (A) 'Panhandle' model, which proposes a partially complementary double-stranded region formed by the conserved nucleotides at the 5' and 3' ends of the vRNA. (B) 'Fork' model, which predicts a single-stranded conformation encompassing nucleotides 1–10 at the 5' end of the vRNA and nucleotides 1–9 at the 3' end, as well as a double-stranded element for nucleotides 11–13 and 10–12 at the 5' and 3' end of the vRNA, respectively. (C) 'Corkscrew' model, which in contrast to the 'panhandle' and 'fork' models, predicts base-pairing within the 5' or 3' terminal nucleotides, rather than between them. (D) Model combining elements of B and C by proposing base-pairing within the 5' terminal nucleotides, but not within the 3' terminal nucleotides

which the nature of the nucleotide is critical, and region II, in which base-pairing is essential [55].

Polyadenylation signal

Viral mRNA synthesis initiates at the 3' end of the vRNA, but in contrast to cRNA synthesis, does not yield a full-length transcript; rather, the viral polymerase complex stops at a stretch of uridine residues near the 5' end of the vRNA. Therefore, the double-stranded promoter region II was thought to halt the polymerase complex to allow polyadenylation of the nascent transcript at the uridine residues. To test this model, two research

groups inserted additional nucleotides between the promoter and the uridine stretch, or interrupted the uridine stretch by inserting nonuridine nucleotides [56,57]. These studies demonstrated that an uninterrupted uridine stretch adjacent to the double-stranded promoter region II is crucial for polyadenylation. Specific nucleotides in region I that constitute a polymerase binding site at the 5' end of the vRNA were subsequently found to be critical for efficient polyadenylation [58]. Transfected viruses were also used to demonstrate that the polymerase complex 'stutters' at the uridine stretch near the 5' end of the vRNA to cause polyadenylation [59,60]. Poon *et al.* [60] generated a transfected virus whose NA polyU-stretch was replaced with a polyA-stretch, resulting in polyuridylated NA-mRNA. The polyuridylated mRNA was largely retained in the nucleus, indicating that a polyA-tail is important for efficient nuclear export of viral mRNAs [60].

Segment-specific noncoding regions

The segment-specific noncoding regions that lie between the promoter and the start or stop codon vary among the segments in their length and their nucleotide composition and were therefore thought to harbour regulatory signals. The generation of transfected viruses with deletions, insertions, or mutations in the noncoding regions proved that some of these segments are dispensable for viral replication [61–64]. However, for some of the transfected viruses, the amounts of the respective vRNAs were altered [63,64], suggesting that the noncoding regions contain signals that regulate replication and/or transcription.

Neuraminidase gene

Enami *et al.* [34] were the first to generate an influenza virus encoding an altered viral protein. The neuraminidase protein of A/WSN/33 (H1N1) virus confers trypsin-independent virus growth in MDBK cells. This group transfected *in vitro* generated A/WSN/33 NA vRNPs into cells that had been infected with a reassortant virus that relies on trypsin for its growth. Thus, in the absence of trypsin, virus containing the *in vitro* generated A/WSN/33 NA gene segment had a growth advantage and outgrew the trypsin-dependent helper virus. By contrast, Liu and Air [65] used a mutant helper virus encoding a truncated NA protein to rescue full-length NA.

For influenza B virus, strain-specific anti-NA antibodies allowed the generation of transfecant viruses with alterations in their NA protein [66].

The neuraminidase (NA) protein prevents virion aggregation and facilitates virus release from infected cells by removing sialic acid from sialyloligosaccharides on cell surface receptors and HA and NA proteins. In contrast to human virus NAs, all avian NA proteins have high levels of haemadsorption activity. To investigate the biological significance of this activity, Kobasa *et al.* [67] generated a transfecant avian virus without the NA haemadsorption activity. The mutant virus replicated as efficiently as wild-type virus in ducks, so that the biological significance of the high adsorption activity of avian NAs remains elusive.

Researchers also generated transfecant viruses to obtain insight into the structural features of NA. The NA of A/WSN/33 virus, which is neurovirulent in mice, differs from all other NAs by the lack of a glycosylation site on its globular head. A mutant virus with a glycosylation site at this position did not replicate in mouse brain [68], indicating that the glycosylation site is critical for neurovirulence in these animals.

The globular head with its enzymatic centre is connected to the viral envelope by a stalk that is highly variable among the nine influenza A NA subtypes. Studies with transfecant viruses with a deleted stalk or insertions in the stalk revealed that the stalk is not required for virus replication, but affects the replicative efficiency of the virus [69–72]. In contrast to the stalk, the short cytoplasmic NA tail is highly conserved. Replacement of the tail with the corresponding amino acids of the influenza B virus NA tail produced virus [73,74]; deletion of the entire tail also resulted in viable virus, although it was attenuated and displayed elongated shapes [75,74]. Thus, the NA tail is not essential for virus replication, but affects the formation of virus particles.

Haemagglutinin gene

Transfecant influenza A and B viruses with mutations in their HA proteins can be generated by antibody selection [35,62,76] or host range restriction [77]. HA, the major viral glycoprotein, mediates virus binding to cellular receptors, as well as fusion of the viral and the endosomal membranes. The latter process requires that the

HA precursor (HA0) be post-translationally cleaved by cellular proteases into the HA1 and HA2 subunits. A direct link between HA cleavability and virulence was demonstrated by introducing alterations into the HA cleavage sequence [76,78]: multiple basic residues at the HA cleavage site, which are recognised by ubiquitous proteases (e.g. furin [79]), confer high virulence, resulting in systemic infections. By contrast, local infections in the respiratory and/or intestinal organs are caused by viruses that contain a single basic amino acid at the cleavage site.

The HA cytoplasmic tail is highly conserved among the 15 HA subtypes. It contains three conserved Cys residues that can be palmytated and therefore were thought to be critical for virus assembly. Whereas Jin *et al.* [80] and Lin *et al.* [81] were able to rescue virus whose Cys residues in the HA cytoplasmic tail had been replaced, Zurcher *et al.* [82] failed to do so. Since different HA subtypes were used for these studies, the discrepant results may reflect differences in the ability of the HAs to tolerate replacements. Like its NA counterpart, the HA cytoplasmic tail can be deleted [83]. An HA/NA tail-less mutant had a highly elongated shape [84] and a reduced vRNA-to-protein content [85]. Most likely, the HA tail affects particle formation by interacting with other viral proteins, although it does not appear crucial in normal progression of the viral life cycle.

M and NP genes

M2 is an integral membrane protein that functions as an ion channel, allowing proton influx into the virion in late endosomes. The resulting pH shift causes the dissociation of M1 from RNP complexes, a prerequisite for RNP nuclear import. Late in infection, the M2 ion channel raises the pH in the trans-Golgi network to prevent the acid-induced conformational change of intracellularly cleaved HA. The M2 ion channel activity can be blocked by amantadine. Hence, in the presence of amantadine, transfecant virus encoding an amantadine-resistant M2 gene derived from A/PR/8/34 virus can be selected against the background of helper virus encoding amantadine-sensitive M2 protein [86]. This selection system was used to generate virus that lacked the carboxyl-terminal amino acid of the M2 cytoplasmic tail [86]. In contrast, deletion of the 5 or 10 carboxyl-terminal M2 amino acids did not yield

replicating virus, indicating that these residues play an important role in influenza virus replication [86]. Furthermore, reverse genetics was used to study the role of the highly conserved Cys residues in the M2 protein [87], as well as the significance of Ser64, the primary M2 phosphorylation site [88]. Transfected virus with replacements at Ser64 or at the conserved cysteine residues grew as well as wild-type virus in tissue culture, mice and/or ferrets, demonstrating that these residues are not required for viral replication [87,88]. Yasuda *et al.* [89] took advantage of an M1 temperature-sensitive (*ts*) mutant of A/WSN/33, replacing the M1 with the corresponding sequence of A/Aichi/2/68 virus. Characterisation of transfected viruses revealed that the rapid-growth phenotype of A/WSN/33 virus is determined by its M1 protein [89]. A *ts* mutant also allowed a selection system to be established for the NP gene [38], although it has not yet been used for extensive mutagenesis of NP.

NS gene

A selection system for the NS gene was exploited by Enami *et al.* [90] to produce a virus with nine gene segments: an NS gene segment encoding wild-type NS1, but not NS2 protein, was incorporated into infectious virions by use of a helper virus with a *ts* defect in NS1 [35]. Garcia-Sastre *et al.* [91], also using a *ts* helper virus, generated a transfected virus lacking the NS1 gene (delNS1). The delNS1 virus grows in Vero cells to titres one log unit lower than those obtained for wild-type virus; however, its growth is severely restricted in MDCK cells and embryonated eggs [91]. Considering these growth characteristics and the fact that Vero cells do not synthesise interferon (IFN), Garcia-Sastre *et al.* [91] speculated that the NS1 protein inhibits the IFN-mediated antiviral response of the host. This hypothesis was supported by findings that delNS-virus, but not wild-type virus, both stimulated the expression of a reporter gene under the control of an IFN-regulated promoter [91] and activated NF- κ B, a known transactivator of IFN- β promoters [92]. In addition, Hatada *et al.* [93] observed activation of the interferon-induced dsRNA-dependent protein kinase (PKR) by vRNA in *in vitro* experiments, and found that this activation could be blocked by preincubation of the vRNAs with NS1. The central role of NS1 in suppression of the PKR-mediated antiviral response was

confirmed by Bergmann *et al.* [94] in *in vivo* experiments. Further insight into the mechanism by which NS1 counteracts the antiviral defence systems of the host came from a study by Talon *et al.* [95]. These authors found that wild-type influenza virus, but not delNS1 virus, inhibits activation of the interferon regulatory factor 3 (IRF-3), which regulates IFN-alpha/beta gene expression. Despite its critical role in establishing an antiviral state, PKR activity does not seem to be critical for host cell protein suppression in infected cells [96]. Nonetheless, the ability to introduce mutations into the viral genome has enhanced our understanding of the interplay between viral and host cell proteins in IFN-mediated antiviral responses [97].

PB2 gene

Using the knowledge that PB2 affects the host range of influenza virus, Subbarao *et al.* [98] established a selection system for this protein. An avian-human reassortant virus (containing an avian virus PB2 gene in a human viral gene background) that grows poorly in mammalian cells was used as a helper virus to rescue a human virus PB2 gene that confers a growth advantage in these cells. With this approach, researchers introduced mutations into the PB2 gene that caused temperature-sensitive and attenuated phenotypes [98,99]. To restrict a reversion to the wild-type genotype, Subbarao *et al.* [99] and Parkin *et al.* [100,101] took these studies one step further and introduced several mutations into the PB2 gene.

Expression of foreign polypeptides or proteins

To explore the utility of influenza viruses as a vaccine vector, different research groups generated transfected viruses expressing foreign polypeptides [reviewed in 102]. Viruses were constructed that expressed short foreign polypeptides integrated into one of the viral glycoproteins [72,103–111]. Examples include the introduction of a cytotoxic T lymphocyte-specific epitope of the LCMV nucleoprotein in the NA stalk [72], the introduction of the V3 loop of HIV-1 gp120 protein or a highly conserved epitope from the ectodomain of HIV-1 gp41 into the antigenic site B of A/WSN/33 HA [104,106,108], or the replacement of the antigenic site B of A/WSN/33 (H1N1) with the corresponding sequence of H3 HA [103]. In these cases, the transfected viruses induced immune responses against the foreign epitopes.

Various approaches were taken to construct transfectant viruses expressing full-length foreign proteins. Garcia-Sastre *et al.* [112] generated a bicistronic vRNA with an internal ribosomal entry site (IRES). In their construct, the foreign protein was expressed via cap-mediated initiation of translation, whereas the viral NA protein was translated from the second open reading frame via internal binding of the ribosome, mediated by the IRES [112]. In contrast, Percy *et al.* [113] inserted the 17 amino acid 'protease 2A' sequence from foot-and-mouth disease virus between the reporter protein CAT and NA, although this sequence may serve as an IRES instead of a protease [114]. Thus, a polyprotein was generated and post-translationally cleaved to release NA and a CAT-protease 2A fusion protein.

Are additional gene segments stably maintained?

Further studies of influenza virus as a vector for gene delivery investigated the stability of additional gene segments encoding a gene of interest. Initial experiments by Luytjes *et al.* [33] indicated that a virus-like reporter gene construct could be maintained for only about three passages in cells. However, Neumann *et al.* [46] described a promoter mutant (G3A, U5C, C8U mutations at the 3'end of the vRNA) capable of increasing reporter gene expression 20-fold. Most likely because of their preferential transcription and/or replication, reporter-gene segments containing these mutations in their promoter region were stably maintained through several rounds of virus propagation. Prompted by this finding, Zhou *et al.* [115] generated an influenza virus possessing an additional segment that expresses a hybrid protein composed of the ectodomain of the E2 glycoprotein of classical swine fever virus and the transmembrane domain and cytoplasmic tail of influenza HA. The protein coding region was flanked by the HA noncoding regions, which contained the above-described promoter mutations. The additional gene segment was maintained during 11 passages, and immunogold labelling performed after the second passage revealed that 16% of the viruses expressed the fusion protein [115]. Thus, recombinant viruses can be generated that maintain additional segments encoding foreign proteins through multiple rounds of virus propagation.

Limitations of helper virus-dependent reverse genetics
The RNP transfection system and the subsequently developed systems for the generation of transfectant viruses were a par force ride at the time and for the first time allowed the introduction of mutations into the influenza viral genome. Still, these systems had limitations. The purification of RNP complexes, their reconstitution with *in vitro* synthesised RNA, and transfection of the resulting vRNPs were all technically demanding, and the efficiency of transfectant virus generation was very low, requiring strong selection systems. Moreover, the limited number of selection systems curtailed motivation to alter the viral genome, leading to efforts to secure means of modifying any viral segment without technical limitations.

SYSTEM FOR THE MUTAGENESIS OF ANY INFLUENZA VIRUS SEGMENT

Enami *et al.* [116] established a system that allowed the alteration of any viral segment. Briefly, purified RNPs were incubated with a cDNA hybridising to the target gene segment. The formed RNA-DNA hybrid was digested by RNase H, yielding RNP devoid of the target vRNA. The depleted RNPs were then mixed with artificially generated vRNPs for the gene of interest. In contrast to previously published methods, this approach allows the generation of transfectant virus without the need for selection systems. Enami and Enami [117] used this system to generate recombinant influenza virus with deletions in either the N-terminal or C-terminal region of the NS1 protein. Both viruses were attenuated and the former exhibited reduced levels of protein synthesis for all viral proteins. Deletion of the C-terminal region of NS1, in contrast, reduced the level of M1, but not of NP protein expression, indicating that NS1 functions in the translational stimulation of M1. However, the system remained technically challenging.

GENERATION OF INFLUENZA VIRUS ENTIRELY FROM CLONED cDNA

In 1999, a decade after the inception of influenza virus reverse genetics, Neumann *et al.* [118] (Figure 3), as well as Fodor *et al.* [119], generated influenza A virus entirely from cloned cDNAs.

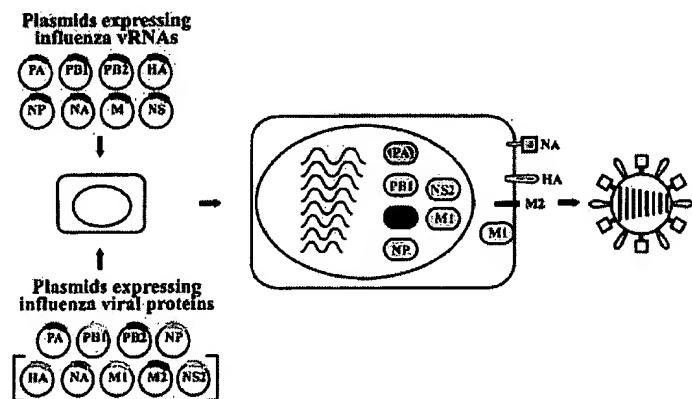


Figure 3. Generation of influenza virus entirely from cloned cDNAs. Eight plasmids that contain the coding region for one of the viral segments, flanked by RNA polymerase I promoter and terminator sequences, are transfected into eukaryotic cells for vRNA synthesis by RNA polymerase I. Nine plasmids for the expression of all viral structural proteins or only four plasmids for the viral proteins required for replication and transcription of the vRNA (i.e. the polymerase and NP proteins) (enclosed by a bracket) are provided by cotransfection of cells with protein expression constructs. Thus, the influenza viral replication cycle is initiated, yielding $>10^7$ artificially generated viruses per mL of cell culture supernatant

RNA polymerase I system for the generation of influenza A virus from cloned cDNAs: negative-sense approach

Neumann *et al.* [118] cloned cDNAs encoding all eight vRNAs of A/WSN/33 virus between the human RNA polymerase I promoter and mouse RNA polymerase I terminator. Transfection of the resultant plasmids into 293T cells yielded vRNA synthesised by cellular RNA polymerase I. When cells were cotransfected with protein expression plasmids for all viral structural proteins, 8×10^7 infectious viruses per mL of supernatant were obtained. Thus, the once-unattainable goal of generating influenza virus 'from scratch' was achieved. The proposed system was not complex, as it required only DNA cloning, DNA purification and DNA transfection techniques — methods widely available in molecular biology and virology laboratories. Infection with helper virus was no longer required, thus circumventing the need for cumbersome selection of transfector viruses.

In the initial experiments, cells were cotransfected with plasmids expressing all nine structural proteins. Despite the introduction of 17 plasmids into cells (8 for RNA segments and 9 for structural protein expression), this approach yielded more than 10^7 infectious viruses per mL of supernatant. More recently, we have been able to produce more than 10^8 infectious particles per mL of the supernatant by expressing only four proteins required

for viral RNA transcription and replication (i.e. PB2, PB1, PA, and NP, unpublished data). Despite its requirement for 12 plasmids, the RNA polymerase I system for influenza virus generation is among the most efficient systems for the generation of negative-sense RNA viruses. This can be attributed to the high efficiency of 293T cells transfection, and the resultant pool of cells containing the full complement of constructs required to initiate virus replication. Furthermore, RNA polymerase I is abundantly expressed in growing cells, thus ensuring efficient replication of RNA polymerase I constructs.

Fodor *et al.* [119] reported a similar approach in which influenza virus was generated by cloning cDNAs encoding vRNAs under control of the human RNA polymerase I promoter. The hepatitis delta virus ribozyme was used to generate the 3' ends of the viral transcripts. Transfection of the RNA polymerase I/ribozyme plasmids into Vero cells, together with protein expression plasmids for the polymerase and NP proteins, yielded infectious virus.

RNA polymerase I system for the generation of influenza A virus from cloned cDNAs: positive-sense approach

A critical requirement for the generation of most nonsegmented negative-sense RNA viruses is the use of a plasmid encoding the antigenomic,

positive-sense RNA, rather than the genomic negative-sense RNA [for a review see [120,121]. The likely explanation is that the antigenomic positive-sense RNA, in contrast to the genomic negative-sense RNA, would not be able to hybridise with positive-sense mRNAs for the nucleoprotein and the polymerase proteins in the cytoplasm of transfected cells. Generation of Sendai [7] and human parainfluenza virus type 3 [9] has also been reported with initiation of the infectious cycle from negative-sense genomic RNA; however, the efficiencies were significantly lower than results with positive-sense RNA.

To determine the efficiency of influenza virus generation from antigenomic positive-sense RNA, we (unpublished data) and others [122] cloned cDNAs encoding all eight cRNAs between the RNA polymerase I promoter and terminator. Transfection of the resulting plasmids thus leads to cRNA synthesis by RNA polymerase I. Cotransfection with protein expression plasmids for all viral structural proteins yielded up to 2×10^7 infectious viruses per mL of supernatant, only slightly lower than the virus titre obtained by expression of vRNA. Thus, in contrast to non-segmented negative-sense RNA viruses, influenza virus can be generated at reasonably high efficiencies from either genomic or antigenomic RNA.

RNA polymerase I/II system for the generation of influenza virus

The RNA polymerase I system for the generation of influenza A virus requires the cotransfection of 12–17 plasmids and may limit the efficient generation of virus to cell lines that can be transfected with high efficiencies. To reduce the number of plasmids, Hoffmann *et al.* [123,124] explored a different route, cloning cDNA encoding vRNA in positive orientation between an RNA polymerase II promoter derived from cytomegalovirus and a polyadenylation signal. They inserted this cassette in negative orientation between RNA polymerase I promoter and terminator sequences. Thus, transfection of the plasmid into eukaryotic cells yields negative-sense vRNA (synthesised by RNA polymerase I) and positive-sense mRNA for protein expression (synthesised by RNA polymerase II) from one template. Consequently, cotransfection of separate protein expression plasmids is no longer necessary, thereby reducing the number of plasmids required for virus generation. The

efficiency of virus generation in this system is comparable to that of the RNA polymerase I system, although the fact that both protein expression and vRNA synthesis are achieved from the same template reduces the flexibility of the system. For example, the RNA polymerase I/II system is not suitable for the formation of virus-like particles lacking or containing lethal mutations in one or more viral segments in studies of viral protein functions or gene delivery (see below). Nonetheless, both the RNA polymerase I and the RNA polymerase I/II systems provide excellent tools for numerous applications.

PERSPECTIVES ON REVERSE GENETICS APPLICATIONS

With the RNA polymerase I system and its modifications in place, tools are now available to study the viral life cycle at the molecular level, to exploit influenza virus as a vector, and, perhaps more importantly, to design attenuated live virus vaccines.

Characterisation of influenza virus proteins and their role in the viral life cycle

In 1918/1919, the 'Spanish Flu' killed 20–40 million people worldwide. Efforts are now under way to determine the genomic sequence of the 1918 influenza virus from formalin-fixed and frozen tissue samples. Basler *et al.* [125] determined the sequence of the NS coding region from a frozen tissue sample, and phylogenetic analyses placed the NS coding region within and near the root of the human/swine group of influenza A viruses. To better understand the high pathogenicity of the 1918 virus, Basler *et al.* [125] used reverse genetics to generate recombinant viruses containing the 1918 NS1 coding region, or both the 1918 NS1 and NS2 coding regions in an A/WSN/33 genetic background. The recombinant viruses, in contrast to the parental A/WSN/33 virus, did not kill mice. Thus, the NS1 and/or NS2 proteins may not have played a major role in the unique pathogenicity associated with the 1918 influenza virus; however, gene constellation effects that may have suppressed a pathogenic potential of the 1918 NS1 and/or NS2 proteins can not be ruled out. Even though the authors did not find a correlation between the NS gene and the high pathogenicity of the 1918 virus, their approach

demonstrates the capability and effectiveness of reverse genetics.

In 1997, an avian influenza virus of the H5N1 subtype was transmitted to humans in Hong Kong, resulting in 18 infected individuals, six of whom died. When tested in a mouse model, the H5N1 isolates from humans were divided into two virulence groups, which correlated with the severity of disease in adult patients from which the viruses were isolated [126,127]. To determine the molecular basis for this difference in virulence, we reproduced a Hong Kong virus lethal to mice and one that is nonpathogenic in mice entirely from cloned cDNA, using the plasmid-based reverse genetics system. A mutation at position 627 in the PB2 viral protein, likely introduced during replication of the virus in humans, determined the outcome of Hong Kong H5N1 viral infection. Moreover, high cleavability of the HA protein, a key determinant of influenza virus pathogenesis in birds, was also an essential requirement for lethal infection in mammals. Thus, these findings solve the molecular basis for pathogenesis of the viruses isolated during the Hong Kong outbreak in 1997 [128].

Among the viruses isolated during the Hong Kong outbreak in 1997, Hoffmann *et al.* [129] reported an H6N1 virus (A/teal/HK/W312/97) that shows >98% homology to the human A/Hong Kong/156/97 (H5N1) virus. Using the RNA polymerase I/II system, Hoffmann *et al.* [124] generated a reassortant virus containing the A/WSN/33 (H1N1) HA and NA genes in the A/teal/HK/W312/97 genetic background and found that the reassortant virus grew to lower titres than its parents, thus exemplifying the importance of the gene constellation.

Reverse genetics has also been used to address the contribution of viral proteins to the viral life cycle. The M2 protein functions as an ion channel and was thought to be essential for viral replication. To the contrary, Watanabe *et al.* [130] generated recombinant viruses lacking or containing a mutation in the M2 transmembrane domain that forms the ion channel. Although the mutant viruses replicated in cell culture, they were attenuated in mice, indicating that M2 ion channel activity is required for full replicative potential.

The NS2 protein had been suggested to function as a viral nuclear export factor since it contains a classical nuclear localisation signal (NES) in its

N-terminus and mediates nuclear export when cross-linked to a reporter protein [131]. Using reverse genetics, Neumann *et al.* [132] demonstrated the nuclear export function of NS2 in the context of viral infection. In cells infected with particles that lacked NS2 or encoded an NS2 with an altered NES, vRNP complexes were retained in the nucleus, indicating that the NS2 NES is critical for viral replication. Taken together, these experiments demonstrate the potential of reverse genetics for deciphering the functions of viral proteins and their contributions to viral pathogenicity.

Attenuated live virus vaccines

Currently, inactivated vaccines generated by conventional reassortment techniques are widely used to prevent or attenuate influenza virus infection. Thus, a high-yielding strain such as A/PR/8/34 is coinfecting with a current epidemic virus. A reassortant is then selected that contains the HA and NA antigens of the wild-type virus and the remaining genes from the high-yielding virus. Reverse genetics could speed this process by generating a set of plasmids encoding the internal genes of a high-yielding virus. After cloning of the HA and NA genes of the currently circulating virus, reassortants could be generated without cumbersome selection procedures.

A cold-adapted live-attenuated vaccine is now in clinical trials [133]. Although providing superior protection over conventional inactivated vaccines in young children, the efficacy of the vaccine in adults is similar to that of inactivated virus [134, reviewed in 135]. Moreover, it contains only a limited number of amino acid replacements [136–138], thus carrying the potential risk of the emergence of revertant virus upon use in a larger population. The new systems for virus generation could be exploited to design a 'master' vaccine strain with multiple attenuating mutations in the genes encoding internal proteins (Figure 4). These mutations might include a deletion in the NS1 gene, such as that resulting in highly attenuated viruses which protected mice from challenge with wild-type virus [139]. In the event of a probable influenza outbreak, the genes encoding the influenza virus surface proteins could be combined with the so-called 'master' genes for the rapid production of attenuated live virus vaccines.

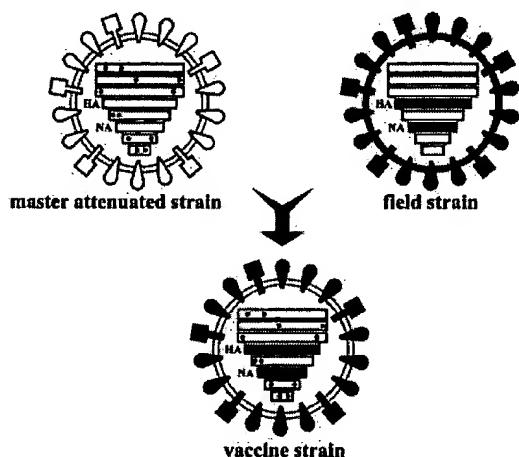


Figure 4. Generation of attenuated live virus vaccines. Reverse genetics can be used to develop an attenuated master virus that contains several mutations in the genes encoding internal proteins. These mutated gene segments can then be combined with the HA and NA genes of a current epidemic strain for vaccine production

Influenza virus vector systems

The ability to artificially generate influenza viruses opens new opportunities to create viruses or replication-incompetent particles for gene delivery. By expressing all viral (structural) proteins and an influenza virus-like RNA encoding a reporter gene, Mena *et al.* [140] devised a method for producing virus-like particles, whose efficiency was later improved [141,142]. With this system, one can now generate virus-like particles that contain vRNAs encoding the proteins required for replication and transcription (i.e. the polymerase and NP proteins), as well as the protein of interest. These particles are infectious and would deliver the gene of interest into target cells. Importantly, owing to the lack of vRNAs encoding structural proteins, no infectious progeny viruses would be formed, ensuring the biological safety of gene delivery. Furthermore, the availability of 15 HA and 9 NA subtypes and their variants would allow the repeated administration of virus-like particles. We have demonstrated the potential of this approach by generating virus-like particles lacking the M and/or NS genes or possessing lethal mutations [130,132]. These VLPs infected cells and viral infection proceeded until the functions of the missing proteins were required, thus ensuring the lack of virus production in VLP-infected cells.

Generation of viruses other than influenza A virus

Successful use of the RNA polymerase I system for influenza A virus generation by several groups underscores its potential for the generation of viruses other than influenza A virus. The most obvious application would be the generation of influenza B and C viruses, or Thogotoviruses, which also belong to the *Orthomyxoviridae* family.

Thogotovirus is a tick-transmitted member of the family *Orthomyxoviridae*. In contrast to influenza A virus, its genome is composed of six segments of negative-sense RNA that encode the three polymerase proteins PB2, PB1 and PA, the nucleoprotein NP, the matrix protein M, and a surface glycoprotein, GP. The GP protein lacks homology to the influenza virus HA but shows similarity to the baculovirus glycoprotein. Interestingly, Thogotovirus does not encode proteins homologous to the influenza virus NS2 or NS1 proteins, which execute critical functions, such as RNP nuclear export or suppression of the interferon-response in influenza virus-infected cells. By combining the vaccinia virus-driven T7 RNA polymerase system and the RNA polymerase I system, Wagner *et al.* [143] succeeded in generating infectious Thogotovirus. 293T cells were first infected with recombinant vaccinia virus expressing T7 RNA polymerase. These cells were then cotransfected with six RNA polymerase I constructs for the synthesis of all viral RNAs, and with six protein expression plasmids for the generation of the viral structural proteins under control of the T7 RNA polymerase promoter. This approach resulted in the generation of up to 10^7 pfu per mL of supernatant and marks the second generation of an orthomyxovirus entirely from cloned cDNA.

Segmented negative-sense RNA viruses also encompass the families *Bunya-* and *Arenaviridae*, which contain three or two RNA segments, respectively. In contrast to influenza virus, members of the *Bunya-* and *Arenaviridae* families replicate in the cytoplasm. Since RNA polymerase I transcripts are synthesised in the nucleolus, it was questionable whether the RNA polymerase I system would allow the generation of bunya- or arenaviruses from cloned cDNAs. Nonetheless, Flick *et al.* [144] reported nuclear export of bunya virus-like transcripts synthesised by RNA

polymerase I, suggesting that this system might also be extended to Bunya- and Arenaviridae family members.

CONCLUSION

Influenza virus reverse genetics has reached a level of sophistication where one can confidently generate virus entirely from cloned DNAs. The new systems makes it feasible to study the molecular mechanisms of virus replication and pathogenicity, as well as to generate attenuated live virus vaccines, gene delivery vehicles, and possibly other RNA viruses from cloned cDNAs. During the next decade, one can anticipate the translation of influenza virus reverse genetics into biomedically relevant advances.

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Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments

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Abstract

A reverse genetics system for the generation of influenza virus A/PR/8/34 (NIBSC vaccine strain) from plasmid DNA was developed. Upon transfection of eight bidirectional transcription plasmids encoding the gene segments of A/PR/8/34 into 293T cells, virus titers in the supernatant were about 10^4 TCID₅₀/ml. The production of A/PR/8/34 in 293T cells was compared to that of A/WSN/33, for which virus titers in the supernatant were 10^7 – 10^8 TCID₅₀/ml. Time-course analysis of virus production indicated that the differences in virus titers were due to reinfection of 293T cells by A/WSN/33 but not A/PR/8/34. Indeed, virus titers of A/PR/8/34 comparable to those of A/WSN/33 were achieved upon addition of trypsin to the culture medium of transfected cells. The production of chimeric viruses revealed that the difference in virus titers between A/PR/8/34 and A/WSN/33 are determined primarily by differences in the surface glycoproteins hemagglutinin and neuraminidase and the polymerase protein PB1. In conclusion, high-titer virus stocks of recombinant influenza A/PR/8/34 virus can be produced as well as virus stocks with much lower titers, but without the requirement of virus amplification through replication.

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1. Introduction

For a long time the fundamental research of influenza A viruses has been hampered by the lack of availability of efficient reverse genetics systems. Although the earliest reverse genetics techniques for negative stranded RNA viruses were in fact developed for influenza A virus (Enami et al., 1990; Luytjes et al., 1989), the rescue of this virus exclusively from recombinant DNA was achieved only recently (Fodor et al., 1999; Neumann et al., 1999). Recombinant influenza virus was produced upon transfection of eukaryotic cells with a set of eight plasmids from which each of the genomic viral RNA (vRNA) segments was transcribed by RNA polymerase I and a set of four additional plasmids expressing the nucleoprotein (NP) and the polymerase proteins PB1, PB2, and PA. The reported efficiencies of virus production using these 12-plasmid systems were relatively low with less than 10^4 plaque-forming units (pfu) of

influenza virus A/WSN/33 per ml of transfected cell supernatant. Neumann et al. reported that upon co-expression of five additional plasmids encoding the hemagglutinin (HA), neuraminidase (NA), matrix proteins 1 and 2 (M1 and M2) and non-structural protein 2 (NS2), virus titers in the supernatants could be increased up to 5×10^7 pfu/ml. An elegant modification of these 12 and 17-plasmid systems came from Hoffmann et al. who implemented bidirectional vectors to reduce the number of transfected plasmids to eight. With this system, the negative-stranded vRNA and the positive-stranded mRNA can be synthesized from the same plasmid and virus titers up to 2×10^7 were reported (Hoffmann et al., 2000). The ability to produce recombinant influenza A virus rapidly and at such high titers will greatly facilitate future influenza virus research. Indeed, several influenza virus strains have now been produced from recombinant DNA to address a number of fundamental research questions in the influenza virus field (Hatta et al., 2001, 2002). In addition, these techniques may be used to produce "conventional" vaccine viruses and to design live attenuated vaccines through genetic engineering. Finally, the use of influenza A viruses as gene delivery vectors and

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to express foreign proteins of interest may now be employed.

It is important to note that the reverse genetics systems described above are all based on influenza virus A/WSN/33 (H1N1). Although influenza virus A/WSN/33 has been used successfully to address many research questions, the surface glycoproteins of this virus have properties that may be undesirable for certain purposes. The NA of A/WSN/33 can bind plasminogen that upon conversion to plasmin can cleave the HA to yield functional HA₁ and HA₂ subunits (Goto and Kawanka, 1998). As a result, the virus can replicate without trypsin in *in vitro* cell cultures. For certain purposes, such as mutagenesis studies, virus replication in the transfected producer cells may be undesirable since reverse mutations and second-site mutations may occur. Moreover, for the generation of reassortant viruses to be used as vaccines, influenza virus A/PR/8/34 has been the strain of choice for many years. Therefore, we have designed a reverse genetics system to produce recombinant influenza virus A/PR/8/34. We have used both the 12-plasmid and 8-plasmid systems and compared the virus production of A/PR/8/34 with A/WSN/33. We conclude that virus titers of $\sim 10^4$ can be obtained without virus replication in the transfected cell culture which can be boosted to $>10^7$ when the virus is allowed to replicate. This reverse genetics system may thus be useful for research purposes as well as for the production of vaccine virus.

2. Materials and methods

2.1. Cells and viruses

Madin–Darby Canine kidney (MDCK) cells were cultured in EMEM (BioWhittaker) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate, 10 mM Hepes and non-essential amino acids. 293T cells were cultured in DMEM (BioWhittaker) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and non-essential amino acids.

Influenza virus A/PR/8/34 was kindly provided by Dr. Wood, the National Institute for Biological Standards and Control, Potters Bar, United Kingdom. Because this strain is adapted for replication in embryonated chicken eggs and may not replicate optimally in mammalian cell cultures, this virus was passaged seven times at a low multiplicity of infection in MDCK cells grown in Episert media (Gibco BRL) supplemented with 10 IU/ml penicillin and 10 µg/ml streptomycin. After the seventh passage virus titers of 10^8 TCID₅₀/ml were obtained routinely.

2.2. Transfection of 293T cells

Transient calcium phosphate-mediated transfections of 293T cells were performed essentially as described (Pear

et al., 1993). Cells were plated the day before transfection in gelatinized 100 mm diameter culture dishes to obtain 50% confluent monolayers. After overnight transfection with 25–50 µg plasmid DNA, the transfection medium was replaced with fresh medium supplemented with 2% FCS for virus production or 10% FCS for all other transfections. Cells were incubated for 30–72 h, after which supernatants were harvested and cells were analyzed for fluorescence if appropriate. Plasmid pEGFP-N1 (Clontech, BD Biosciences, Amsterdam, The Netherlands) was transfected in parallel in all experiments and the percentage of fluorescent cells was measured in a FACScan, confirming that the transfection efficiency ranged from 95 to 100%. Virus-containing supernatants were cleared by centrifugation for 10 min at 300 × g. Virus titers in the supernatant were determined either directly or upon storage at 4 °C for less than 1 week, or at –80 °C for longer than 1 week.

2.3. Plasmids

Plasmids pPOLI-CAT-RT, pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP were a kind gift from Drs. Garcia-Sastre and Palese (Mount Sinai School of Medicine, New York, USA), plasmids pH1863 and pH2428 from Dr. Hobom (University of Giessen, Germany) and plasmids pHW2000, and pHW181 through pHW188 from Dr. Webster (St. Jude Children's Research Hospital, Memphis, TN, USA).

The human RNA polymerase I promoter (Phu) was amplified by PCR using plasmid pH1863 as template and cloned in plasmid pSP72 (Promega Benelux, Leiden, The Netherlands) using XbaI and XbaI sites included in the primers. The murine RNA polymerase I terminator or the hepatitis delta ribozyme sequence were amplified by PCR using plasmids pH2428 or pPOLI-CAT-RT as templates and cloned in pSP72-Phu using XbaI and BamHI sites present in the primers to give plasmids pSP72-PhuTmu and pSP72-PhuThep, respectively. Flanking the XbaI site between the Phu and Tmu or Thep sequences we included BpuAI sites to enable the forced directional cloning of influenza A virus cDNAs in these vectors. The eight genomic segments from influenza virus A/PR/8/34 were amplified by RT-PCR and cloned in pSP72-PhuThep (segments 2 and 6) or pSP72-PhuTmu (all other segments). Vector pSP72-PhuThep was used for segments 2 and 6 because pSP72-PhuTmu did not yield the desired recombinant plasmids for unknown reasons. Of note, vectors pSP72-PhuTmu and pSP72-PhuThep work equally well in transient assays in 293T cells (data not shown). To generate bidirectional expression vectors, plasmid pHW2000 was modified so that the BsmBI sites were at the same positions relative to the RNA polymerase I promoter and terminator sequences as the BpuAI sites in our own constructs. The eight genomic segments from influenza virus A/PR/8/34 were subsequently amplified by PCR and cloned in this modified pHW2000 vector. All plasmids were sequenced using a Dye-terminator ET

terminator sequencing kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and an ABI 373 automatic DNA sequencer (PE Biosystems), according to the instructions of the manufacturer. All PCR primer sequences and plasmid maps are available on request.

2.4. Virus titration

Virus titrations were performed as described previously (Rimmelzwaan et al., 1998). Briefly, 10-fold serial dilutions of the transfected cell supernatants were prepared in infection medium. Infection medium consisted of EMEM (BioWhittaker) supplemented with 4% bovine serum albumine (fraction V, GibcoBRL), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate, 10 mM Hepes, non-essential amino acids, and 25 µg/ml trypsin. Prior to inoculation, the cells were washed twice with PBS. One hundred milliliter of the diluted culture supernatants was used to inoculate a confluent monolayer of MDCK cells in 96 wells plates. After 1 h at 37 °C the cells were washed again with PBS and 200 µl fresh infection medium was added to each well. At 3 days post infection, the supernatants of these cultures were tested for HA activity as an indicator for infection of the cells in individual wells. The titers of infectivity were calculated from 10 replicates according to the method of Spearman–Karber.

3. Results

3.1. A reverse genetics system for influenza virus A/PR/8/34

Influenza virus A/PR/8/34, obtained from the National Institute for Biological Standards and Control, United Kingdom, was passaged seven times in MDCK cells in the presence of trypsin. This virus was found to replicate

to high titers in embryonated eggs and MDCK cell cultures (data not shown). The eight genomic segments of this virus were amplified by PCR and cloned into plasmids pSP72-PhuTmu or pSP72-PhuThep. Each of the eight plasmids was sequenced and the sequences were compared with those of A/PR/8/34 and other influenza A virus sequences available from the Influenza Sequence Database (<http://www.flu.lanl.gov>) (Table 1). For the total genome of A/PR/8/34 which is 13 588 nucleotides in length, we found 111 nucleotide substitutions as compared to sequences of A/PR/8/34 available from the database, resulting in 39 amino acid substitutions. This is not surprising since the passage history of the two A/PR/8/34 strains may be quite different. Analysis of all the sequences available from the Influenza Sequence Database revealed that of these 39 amino acid substitutions only one was unique to A/PR/8/34.

The eight constructs encoding the gene segments of A/PR/8/34 were transfected into 293T cells together with expression plasmids for the polymerase proteins and nucleoprotein of influenza virus A/PR/8/34: HMG-PB2, HMG-PB1, HMG-PA, and HMG-NP (Pleschka et al., 1996). At 72 h after transfection, an infectious virus titer of 1×10^3 TCID₅₀/ml of influenza virus A/PR/8/34 was detected in the culture supernatant. However, when this experiment was subsequently repeated five times, virus could be produced only once more with equally low virus titers (data not shown). The inability to generate recombinant virus reproducibly was not due to low transfection efficiencies, since transfections with pEGFP-N1 performed in parallel with these experiments revealed >99% fluorescent cells in these cultures as measured in a FACScan.

In the meantime, a bidirectional 8-plasmid reverse genetics system was described by Hoffmann et al. that we compared with our own 12-plasmid system. To this end, cDNAs encoding the gene segments of influenza virus A/PR/8/34 were cloned into plasmid pHW2000 (Hoffmann et al., 2000).

Table 1
Comparison of nucleotide and amino acid sequences of the genome of MDCK-adapted A/PR/8/34* with those of A/PR/8/34 and A/WSN/33 from the Influenza Sequence Database

Gene segment	Encoded protein	A/PR/8/34			A/WSN/33		
		Accession number	Nucleotide substitutions	Amino acid substitutions	Accession number	Nucleotide substitutions	Amino acid substitutions
1	PB2	NC002023	19	7	J02179	89	26
2	PB1	NC002021	18	8	J02178	62	22
	PB1F2		3	3		10†	8†
3	PA	NC002022	20	1	X17336	65	17
4	HA	NC002017	13	9	J02176	105	54
5	NP	NC002019	15	4	M30746	56	15
6	NA	NC002018	9	5	J02177	82	38
7	M1	NC002016	6	0	L23818	34	5
	M2			2			9
8	NS1	NC002020	11	2	M12597	32	8
	NS2			1			6

* These sequences are available from the Influenza Sequence Database under accession numbers ISDN13419–13426.

† Total insertion counted as a single substitution.

Of note, the sequence of each of the A/PR/8/34 gene segments was identical to that in the 12-plasmid system. Transfection of the eight plasmids encoding the gene segments of influenza virus A/PR/8/34 resulted in a virus titer in the supernatant of $\sim 10^4$ TCID₅₀/ml 30 h post transfection. More importantly, the virus titers obtained in these transfection experiments were highly reproducible. The titers that we obtained upon transfection of constructs encoding the eight gene segments of influenza virus A/WSN/33 into 293T cells were much higher, however, ranging from 10^7 – 10^8 TCID₅₀/ml.

3.2. Effect of mutations at position 4 of the 3' terminus

The eight genomic cDNAs inserted in both the 12-plasmid system and the 8-plasmid system were generated using primers specific for the 12 conserved nucleotides (nt) at the 3' terminus and 13 nt at the 5' terminus. The virus-specific sequence at the 3' terminus was UCGUUUUUCGUCC, despite the fact that for A/PR/8/34 gene segments 1, 2, 3, 6, and 7 the fourth nucleotide position was reported to be C rather than U (according to the Influenza Sequence Database; <http://www.flu.lanl.gov>). To test the effect of mutations at the position 4 nt in the 3' terminus, we generated a set of 8 plasmids containing the eight gene segments of A/PR/8/34 with a C at position 4. The nucleotide sequence of each of these gene segments was identical to the sequence of the original constructs except for this fourth nucleotide. Upon transfection of 8 plasmids with a C at position 4, virus titers were 5.2×10^3 TCID₅₀/ml, which is slightly higher than upon transfection of 8 plasmids with a U at position 4 (mean virus titer of 3.3×10^3 TCID₅₀/ml). We next generated sets of recombinant viruses in which each of the genomic segments was replaced with a segment containing C at position 4. These viruses were all produced at comparable levels. The virus titers obtained upon transfection of plasmids representing "wild type" A/PR/8/34 according to the Influenza Sequence Database, with a U in segments 4, 5, and 8 and a C in segments 1, 2, 3, 6, and 7 resulted in a virus titer of 1.1×10^4 TCID₅₀/ml. From these data we concluded that the low virus titers obtained with A/PR/8/34 were not due to mutations at position 4 in our set of plasmids.

3.3. Increased virus titers due to reinfection

The HA precursor protein (HA₀) of influenza A viruses is cleaved by cellular proteases into HA₁ and HA₂ subunits to yield membrane fusion-competent virus particles. For many *in vitro* cell cultures infected with influenza A virus, trypsin is added to the culture medium to enable cleavage of HA. Influenza virus A/WSN/33 can replicate in cell cultures without the addition of trypsin to the culture medium. The NA of A/WSN/33 can bind plasminogen that is converted to plasmin, which can subsequently cleave the HA₀ into functional HA₁ and HA₂ subunits (Goto and Kawaoka, 1998). We therefore wished to test if the differences in virus titers

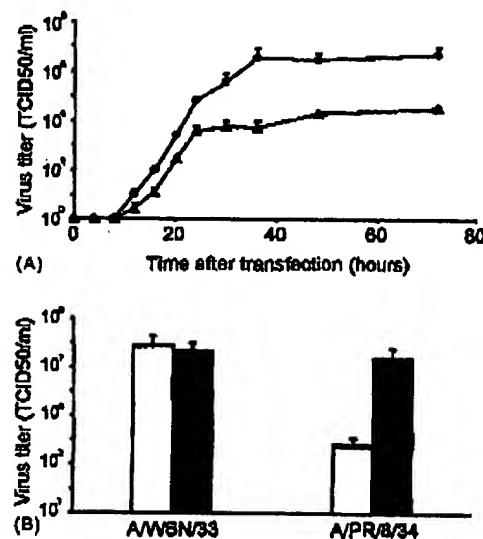


Fig. 1. Virus production in the presence and absence of trypsin. Supernatant of 293T cells transfected with constructs encoding A/WSN/33 (●) or A/PR/8/34 (△) was harvested at different time points after transfection and titrated on MDCK cells (panel A). Average and standard deviation calculated from two independent experiments are shown. After transfection, trypsin was added to the supernatant of 293T cells transfected with constructs encoding A/PR/8/34 or A/WSN/33 (B). Supernatants were harvested 72 h after transfection and titrated on MDCK cells. (White bars) no trypsin and (black bars) with trypsin. Average and standard deviation calculated from three independent experiments are shown.

between influenza viruses A/WSN/33 and A/PR/8/34 could be explained by trypsin-independent replication in the 293T cells by A/WSN/33 but not by A/PR/8/34.

To this end, we first performed time-course analyses of virus production from 293T cells transfected with constructs encoding either influenza virus A/PR/8/34 or A/WSN/33. Influenza viruses A/PR/8/34 and A/WSN/33 were produced at the same rate during the first 24 h of virus production after transfection. However, from 24 h post transfection onwards, virus production from cells transfected with constructs encoding A/PR/8/34 hardly increased, while virus production from cells transfected with constructs encoding A/WSN/33 continued to increase logarithmically until 36 h post infection (Fig. 1A). The A/WSN/33 virus particles produced late after transfection could well be derived from 293T cells infected with virus produced in the early phase.

To gain further evidence that the high influenza virus A/WSN/33 titer could indeed be caused by the trypsin-independent infection of 293T cells by influenza virus A/WSN/33 but not by A/PR/8/34, we next added trypsin to the culture medium after transfection of 293T cells to a concentration of 0.25 mg/ml. Indeed, upon the addition of trypsin, the virus titers of influenza virus A/PR/8/34 were at the same level as those of influenza virus A/WSN/33 (Fig. 1B). These data indicate that the inability of influenza virus A/PR/8/34 to infect 293T cells without the addition

of trypsin to the culture medium can explain relatively low virus titers of recombinant virus produced from 293T cells. By adding trypsin to the culture medium of transfected 293T cells it is possible to produce high titers of influenza virus A/PR/8/34.

3.4. Analysis of A/WSN/33-A/PR/8/34 chimeric viruses

We next wished to address the question whether NA was the sole determinant of the difference in virus titers obtained with influenza virus A/PR/8/34 and A/WSN/33 due to trypsin-independent replication of the latter virus. To this end, chimeric influenza viruses were produced consisting of seven gene segments of influenza virus A/WSN/33 and one of influenza virus A/PR/8/34 and vice versa and virus titers were determined in MDCK cells. Virus titers obtained upon transfection of 293T cells with constructs encoding seven influenza virus A/PR/8/34 gene segments and one influenza virus A/WSN/33 gene segment were lower than those of wild type influenza virus A/PR/8/34, except when segments 2 (PB1) or 7 (M) of A/WSN/33 were used (Fig. 2A). Although PB1 and M could be partially responsible for low A/PR/8/34 virus titers, the titers obtained with A/PR/8/34 and WSN-PB1 or WSN-M were not nearly as high as wild type A/WSN/33. Therefore, it appeared that none of the gene segments of influenza virus A/PR/8/34 were solely respon-

sible for the low virus titers of influenza virus A/PR/8/34 compared to A/WSN/33.

Viruses with seven influenza virus A/WSN/33 gene segments and one gene segment derived from A/PR/8/34 all yielded titers below wild type A/WSN/33 titers. Upon exchange of gene segments 1, 3, 5, 7, and 8 virus titers were reduced by less than one order of magnitude. The exchange of HA and NA gene segments resulted in a 97-fold and 75-fold reduction in virus production, respectively. Influenza virus A/WSN/33 with PB1 of A/PR/8/34 produced a virus titer that was four orders of magnitude lower than that of wild type A/WSN/33 (Fig. 2B).

Since the receptor-binding activity of HA needs to be balanced by the receptor-removing activity of NA, we generated chimeric virus in which the HA and NA genes of influenza viruses A/PR/8/34 and A/WSN/33 were exchanged simultaneously. Supernatants were harvested 72 h after transfection of 293T cells and titrated on MDCK cells. Compared to 293T cells transfected with constructs encoding all eight influenza virus A/WSN/33 gene segments, cells transfected with constructs encoding six influenza virus A/WSN/33 gene segments and HA and NA of influenza virus A/PR/8/34 produced 141-fold less virus. The 293T cells transfected with six influenza virus A/PR/8/34 gene segments and HA and NA of influenza virus A/WSN/33 produced 224-fold more virus than cells transfected with eight constructs encoding influenza virus A/PR/8/34 (Fig. 3). These chimeric viruses thus demonstrated that the viral surface glycoproteins play a significant role in determining the virus titers produced from 293T cells. Since we already showed that PB1 had a significant influence on virus titers produced in 293T cells (Fig. 2), we next exchanged PB1 together with HA and NA. Recombinant influenza virus A/WSN/33 with segments 2, 4 and 6 of A/PR/8/34 yielded extremely low ($34.1 \text{ TCID}_{50}/\text{ml}$) virus titers from transfected 293T cells. The reciprocal exchange of A/PR/8/34 with segment 2, 4, and 6 of A/WSN/33 yielded virus titers of $3.3 \times 10^6 \text{ TCID}_{50}/\text{ml}$, which is in the same range as virus titers obtained with wild type influenza virus A/WSN/33. These data suggest that the differences in virus titers between recombinant influenza virus A/WSN/33

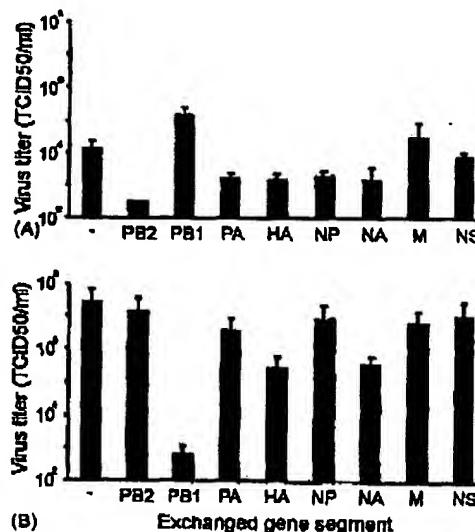


Fig. 2. Production of chimeric influenza viruses from transfected 293T cells. Cells were transfected with seven constructs encoding the gene segments of A/PR/8/34 and one derived from A/WSN/33 as indicated (panel A) or seven gene segments encoding the gene segments of A/WSN/33 and one derived from A/PR/8/34 as indicated (panel B). The first bar in the panel represents wild type A/PR/8/34 (A) or wild type A/WSN/33 (B). Supernatants of transfected cells were harvested 30 h after transfection and titrated on MDCK cells to determine virus titers. Error bars indicate the standard deviation from three independent experiments.

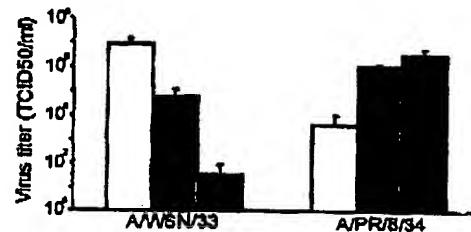


Fig. 3. Virus production upon exchange of HA, NA, and PB1 between A/PR/8/34 and A/WSN/33. Transfected 293T supernatants were harvested at 72 h post transfection and titrated on MDCK cells to determine virus titers. (White bars) wild type virus; (grey bars) exchange of HA and NA; (black bars) exchange of HA, NA, and PB1. Average and standard deviation calculated from four independent experiments are shown.

and A/PR/8/34 are determined primarily by the viral surface glycoproteins and PB1.

4. Discussion

Here, we describe a reverse genetics system for the NIBSC vaccine strain influenza virus A/PR/8/34 adapted to MDCK cells. A bidirectional 8-plasmid transcription system that was first described for influenza virus A/WSN/33 (Hoffmann et al., 2000) was found to be superior to our in-house unidirectional 12-plasmid transcription system. A large difference was observed in the amount of virus produced from 293T cells transfected with constructs encoding influenza virus A/PR/8/34 and A/WSN/33 and the molecular basis for this difference was investigated. The production of A/PR/8/34 could be increased slightly by changing the 3' position 4 nucleotide from a U to a C in the non-coding region (NCR) of gene segments 1, 2, 3, 6, and 7. The NCRs contain the promoter region for transcription and replication, bind the polymerase proteins and are involved in influenza virus packaging. Lee and Seong showed that the 3' position 4 nucleotide in the NCR of NA is involved in the temporal regulation of transcription and replication of NA (Lee and Seong, 1998). If this is true for all gene segments, this could explain the fact that the right combination of Us and Cs in all gene segments can lead to a higher virus yield. It should be noted, however, that the differences in virus titers for our mutant viruses were much smaller than expected from these studies on the NA gene segment.

Our time-course analyses showed that despite similar rates of virus production during the early phase after transfection, virus production of A/PR/8/34 and A/WSN/33 was different after >24 h post transfection. Whereas the amount of virus produced by cells transfected with constructs encoding influenza virus A/PR/8/34 hardly increased from 24 h after transfection onwards, the virus titers in the supernatant of cells transfected with constructs encoding influenza virus A/WSN/33 continued to increase logarithmically (Fig. 1A). Presumably, viruses produced relatively early after transfection were the direct result of transfection of the 293T cells whereas the late production of virus may be explained by the amplification of influenza virus A/WSN/33 by infection of 293T cells.

Indeed, the low titers of influenza virus A/PR/8/34 could be explained by the trypsin-dependent replication of this virus in 293T cells. The addition of trypsin to the culture medium of 293T cells transfected with the constructs encoding influenza virus A/PR/8/34 resulted in virus titers in the supernatant similar to those of cells transfected with constructs encoding influenza virus A/WSN/33 (Fig. 1B).

The construction of PB1 chimeric viruses indicated that this gene has a major influence on the amount of virus produced from 293T cells (Fig. 2). The amino acid sequences of PB1 of influenza viruses A/PR/8/34 and A/WSN/33 are 97.0% identical (Table 1). All but one of the amino acid

residues that are different between PB1 of A/PR/8/34 and A/WSN/33 are found in other strains for which sequences are available from the Influenza Sequence Database, and it is therefore unlikely that these differences have detrimental effects on virus replication. However, A/PR/8/34 has a unique serine residue at position 394 of PB1, which is in the region of PB1 that is involved in binding to cRNA (Gonzalez and Ortin, 1999). Theoretically, this substitution could be (partially) responsible for the low virus titers obtained with A/PR/8/34. However, mutagenesis of this residue in PB1 of A/PR/8/34 did not result in differences in virus titers (data not shown).

PB1 has polymerase activity and binds to PB2 and PA (Gonzalez et al., 1996), vRNA and cRNA (Gonzalez and Ortin, 1999). One of the possible explanations for the effect of PB1 on virus production is that one of these functions is performed better by PB1 of A/WSN/33 as compared to that of influenza virus A/PR/8/34. Another possibility is that the recently discovered peptide PB1F2, encoded by an alternative open reading frame of PB1 is responsible for differences in virus titers. For influenza virus A/PR/8/34 the PB1F2 open reading frame encodes a 87-residue peptide that causes apoptosis (Chen et al., 2001). In A/WSN/33 this open reading frame is also present but is 3 amino acid residues longer than PB1F2 of A/PR/8/34 and different at seven amino acid positions (Table 1). These differences may affect the function of the protein and thereby have an effect on the amount of virus that is produced. However, this is not very likely since Chen et al. could not detect obvious differences in growth ability in eggs, MDCK or MBDK cells between wild type and PB1F2-deficient viruses.

Upon exchange of the HA and NA of A/PR/8/34 with those of A/WSN/33, virus titers in the 293T supernatant were almost as high as those of wild type influenza virus A/WSN/33. When A/WSN/33 had the HA and NA of influenza virus A/PR/8/34 inserted, virus titers dropped but were not as low as those of wild type A/PR/8/34. Upon exchange of PB1 in addition to HA and NA, virus titers were similar to those of wild type viruses. The effect of HA and NA on virus titers is not determined by WSN-NA alone, since WSN-NA in the context of A/PR/8/34 virus did not result in virus titers similar to A/WSN/33 wild type virus. It was shown by Kawaoka et al. that in the 1957 and 1968 pandemic viruses besides HA and NA, PB1 originated from an avian influenza strain (Kawaoka et al., 1989). Also, Hatta et al. were not able to generate reassortant viruses consisting of seven gene segments of A/Mallard/New York/6750/78 and either PB1, PA, HA or NA of A/Memphis/8/88 (Hatta et al., 2002). These data may suggest that PB1, HA and NA can not be easily exchanged between different influenza virus strains.

Our observation that influenza virus A/WSN/33 replicates in cell culture after transfection indicates that care must be taken if this strain is used for mutagenesis studies. Although high virus yields are beneficial for such experiments, undesired mutations may be acquired during these limited

rounds of virus replication. Similarly, cocultivation of transfected 293T cells with MDCK cells or the addition of trypsin to the culture medium may result in increased virus titers, but could also result in reverse mutations and second-site mutations which are undesirable in many studies. Influenza viruses A/Teal/HK/W312/97 (Hoffmann et al., 2000), A/Hong Kong/483/97, and A/HK/486/97 (Hatta et al., 2001), A/Mallard/NY/6750/78 A/Memphis/8/88 (Hatta et al., 2002) were also generated using a 8-plasmid or 12-plasmid reverse genetics system. For efficient generation of high virus stocks of these viruses, however, it was necessary to amplify these viruses in either MDCK cells or embryonated chicken eggs. Recently Schickli et al. and Hoffmann et al. also produced A/PR/8/34 from recombinant DNA using a 12-plasmid and a 8-plasmid system, respectively. Influenza virus A/PR/8/34 reassortant viruses were produced with HA and NA of different influenza A virus subtypes in 293T cells cocultured with MDCK cells (Hoffmann et al., 2002; Schickli et al., 2001). Using a similar approach we have generated A/PR/8/34(NIBSC)-H3N2 reassortant viruses (data not shown), that could be used to generate vaccine strains in the future.

In the reverse genetics system shown here for the NIBSC vaccine strain of influenza virus A/PR/8/34, replication does not appear to occur in 293T cells and it may therefore be the system of choice for some research projects despite the lower virus titers. When needed, e.g. for vaccine virus production, high virus titers of influenza virus A/PR/8/34 can be achieved by adding trypsin to the culture medium of transfected cells, or cocultivation of transfected 293T cells with cells that are more susceptible to virus replication as was also done by Schickli et al. and Hoffmann et al. (Hoffmann et al., 2002).

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Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines

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Summary

Background In response to the emergence of severe infection capable of rapid global spread, WHO will issue a pandemic alert. Such alerts are rare; however, on Feb 19, 2003, a pandemic alert was issued in response to human infections caused by an avian H5N1 influenza virus, A/Hong Kong/213/03. H5N1 had been noted once before in human beings in 1997 and killed a third (6/18) of infected people.^{1,2} The 2003 variant seemed to have been transmitted directly from birds to human beings and caused fatal pneumonia in one of two infected individuals. Candidate vaccines were sought, but no avirulent viruses antigenically similar to the pathogen were available, and the isolate killed embryonated chicken eggs. Since traditional strategies of vaccine production were not viable, we sought to produce a candidate reference virus using reverse genetics.

Methods We removed the polybasic aminoacids that are associated with high virulence from the haemagglutinin cleavage site of A/Hong Kong/213/03 using influenza reverse genetics techniques. A reference vaccine virus was then produced on an A/Puerto Rico/8/34 (PR8) backbone on WHO-approved Vero cells. We assessed this reference virus for pathogenicity in in-vivo and in-vitro assays.

Findings A reference vaccine virus was produced in Good Manufacturing Practice (GMP)-grade facilities in less than 4 weeks from the time of virus isolation. This virus proved to be non-pathogenic in chickens and ferrets and was shown to be stable after multiple passages in embryonated chicken eggs.

Interpretation The ability to produce a candidate reference virus in such a short period of time sets a new standard for rapid response to emerging infectious disease threats and clearly shows the usefulness of reverse genetics for influenza vaccine development. The same technologies and procedures are currently being used to create reference vaccine viruses against the 2004 H5N1 viruses circulating in Asia.

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Introduction

In February, 2003, two family members were admitted to intensive care wards in Hong Kong Special Administrative Region with influenza-like respiratory illness. Avian-like H5N1 influenza viruses were isolated from both patients, one of whom succumbed to infection. This was the first time since 1997 that H5N1 viruses had been identified in human beings, and WHO responded by issuing a pandemic alert. Candidate vaccines were immediately sought. The recent outbreak of severe acute respiratory syndrome (SARS) had been a striking example of the rapid and global spread of an emerging infectious disease. However, even the effects of SARS could be dwarfed by those that could arise with the emergence of an influenza pandemic.

Infection caused by the influenza A virus is a zoonosis, and the animal reservoir of this virus is the aquatic bird populations of the world. The compelling epidemiological link between the presence of the virus in poultry in live-bird markets and the appearance of H5N1 in human beings in 1997 suggested that influenza A viruses can be transmitted directly from avian species to man and can cause severe respiratory disease.^{1–3} Although control of the 1997 outbreak was achieved by culling millions of birds in the Hong Kong markets,⁴ this episode demonstrated that the capability for an effective global response to emerging influenza threats was poor because of technical, legislative, and infrastructural limitations. A disturbing finding that emerged from this event was that the scientific community was unable to produce an effective vaccine even after several years.

The inactivated human influenza vaccines in use today are derived from essentially modified viruses. By exploiting the segmented nature of the influenza A genome, vaccine manufacturers and the laboratories of the WHO influenza network have produced a reassortant virus carrying the circulating virus's gene segments that encode haemagglutinin and neuraminidase, the major targets of neutralising antibodies. The remaining six-gene segments are supplied from PR8, a laboratory-adapted avirulent H1N1 strain.⁵ The resulting reassortant virus has the antigenic properties of the circulating strain and the safety and high-yield properties of PR8.

The first batch of inactivated material against the 1997 H5N1 virus was not ready for clinical trial until 7 months after the second case of human infection arose, and even today the effectiveness of vaccine against this virus has not been proven.⁶ A key reason for this delay in the production of an H5N1-specific vaccine was the nature of the virus itself. The H5N1 virus is highly pathogenic in human beings and poultry. The agent must be handled only under conditions of at least biosafety level 3 (BSL3), and it can kill fertilised chicken eggs, the standard medium for the reassortment and

propagation of influenza virus before its inactivation and formulation for use in vaccines. These same traits are present in the 2003 H5N1 virus.

The pathogenic nature of these H5N1 viruses is linked to the presence of additional basic residues in haemagglutinin at the site of cleavage, a step required for haemagglutinin activation and, thus, for virus entry into cells.⁷⁻⁹ To overcome the high pathogenicity of the virus, polybasic aminoacids have to be eliminated. A rapid, reproducible system to achieve these modifications—ie, plasmid-based reverse genetics—has been developed only in the past 4–5 years.¹⁰⁻¹² The potential benefits of reverse genetics for the generation and attenuation of vaccine candidates against highly pathogenic and low pathogenic influenza viruses are enormous.¹³⁻¹⁵ However, the host specificity of the RNA polymerase I promoter used in the influenza reverse-genetics systems and the required use of an approved cell line limits the practical options for the system's use in the manufacture of human vaccines. The vaccine-candidate reference virus stock described in this report has been produced entirely on a cell substrate licensed for the manufacture of human vaccine, and as such, is—to our knowledge—the first reverse genetically derived influenza vaccine suitable for testing in clinical trials. We describe the construction of a vaccine reference virus in Good Manufacturing Practice (GMP)-grade facilities in less than 4 weeks from the time of virus isolation. Our findings highlight the speed with which new technologies can be implemented in response to influenza pandemic alerts.

Methods

Cells and A/Puerto Rico/8/34 plasmids

We obtained WHO-approved Vero cells (WHO-Vero, X38, p134) from the American Type Culture Collection (Manassas, Virginia, USA). Passage-142 cells (five passages since their removal from a working cell bank) were used for the rescue of the vaccine-candidate virus. The plasmids containing the genes from PR8 have been described elsewhere.¹³

Virus propagation, RNA extraction, PCR amplification, and haemagglutinin and neuraminidase gene cloning
We obtained A/Hong Kong/213/03 (H5N1) that had been passaged in eggs from the WHO influenza network. The virus was isolated and propagated in 10-day-old embryonated chicken eggs. Total RNA was extracted from infected allantoic fluid with use of the RNeasy kit (Qiagen, Valencia, CA, USA) in accordance with manufacturer's instructions. Reverse transcription was carried out with the uni12 primer (5'-AGCA AAAGCAGG-3') and AMV reverse transcriptase (Roche, Indiana Biochemicals Indianapolis, USA). The removal of the connecting peptide of the haemagglutinin was done with use of PCR with the following primer sets: (1) Bm-HA-1 (5'-TATTCGTCAGGGAGCAA AAGCAGGG-3') and 739ΔR (5'-TAATCGTC TCGTTCAATTGAGGGCTATTCTGAGCC-3'); and (2) 739ΔF (5'-TAATCGTCTGTGAAA CTAGAGGATTATTGAGCTATAGC-3') and Bm-NS-890r (5'-ATATCGTCAGGGTGT-3'). We amplified the neuraminidase gene of A/Hong Kong/213/03 using the primer pair Ba-NA-1 (5'-TATTGGTCTC AGGGAGCAAAAGCAGGGT-3') and Ba-NA-1413r (5'-ATATGGTCTCGTATTAGTAGAAACAAG GAGTTTTT-3'). PCR products were purified and cloned into the vector pHW2000 as described previously.¹¹

Rescue of virus from Vero cells

The rescue of infectious virus from cloned cDNA was done under GMP conditions. Vero cells were grown to 70% confluence in a 75 cm² flask, trypsinised (with trypsin-versene), and resuspended in 10 mL of Opti-MEM I (Invitrogen, Carlsbad CA, USA). To 2 mL of cell suspension we added 20 mL of fresh Opti-MEM I; then, we added 3 mL of this diluted suspension to each well of a six-well tissue culture plate (about 1×10⁶ cells per well). The plates were incubated at 37°C overnight. The next day, 1 µg of each plasmid and 16 µL of TransIT LT-1 transfection reagent (Panvera, Madison, WI, USA) were added to Opti-MEM I to a final volume of 200 µL and the mixture incubated at room temperature for 45 min. After incubation, the medium was removed from one well of the six-well plate, 800 µL of Opti-MEM I added to the transfection mix, and this mixture added dropwise to the cells. 6 h later, the DNA-transfection mixture was replaced by Opti-MEM I. 24 h after transfection, 1 mL of Opti-MEM I that contained 1 µg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemicals, Lakewood, NJ, USA) was added to the cells. About 72 h after the addition of TPCK-trypsin, the culture supernatants were harvested and clarified by low-speed centrifugation; we then injected 100 µL of the clarified supernatant into the allantoic cavity of individual 10-day-old pathogen-free embryonated research grade eggs (Charles River SPAFAS, North Franklin, CT, USA).

Pathogenicity testing in chickens

Ten 4-week-old chickens received intravenous injections of 0·1 mL diluted virus (dilution ratio, 1/10). We monitored chickens for signs of disease for 10 days using the Intravenous Pathogenicity Index, approved by the Office of International Epizootics (OIE). Additionally, we took tracheal and cloacal swabs (in 1 mL of media) 3 days and 5 days after infection, and we did assays for the presence of virus by injection of 0·1 mL into all of three 10-day-old embryonated chicken eggs. Haemagglutination activity in the allantoic fluid of these eggs was assessed after incubation at 35°C for 2 days.

Pathogenicity testing in ferrets

We tested pathogenicity of the vaccine in five young adult male ferrets (Marshall's Farms, North Rose, NY, USA) aged 4–8 months (weight about 1·5 kg) that were shown by haemagglutination inhibition assays to be seronegative for currently circulating human influenza A viruses (H3N2, H1N1) and H5N1 viruses. We anaesthetised the ferrets with inhaled isoflurane, and they were then infected intranasally with 10⁶–50% egg infectious dose (EID₅₀)/mL of vaccine reassortant virus or wildtype virus. We monitored the ferrets once per day for signs of sneezing, inappetence, and inactivity, and we recorded rectal temperatures and bodyweights. 3, 5, and 7 days after infection, the ferrets were anaesthetised with ketamine (25 mg/kg), and we collected nasal washes using 1 mL of sterile phosphate-buffered saline (PBS) containing antibiotics. We measured titres of virus in these washes with EID₅₀ assays.

To further assess the pathogenicity of the viruses, we collected tissue samples from lungs, brain, olfactory bulb, spleen, and intestine for virus isolation and histopathological analysis at the time of death or in the case of three ferrets, after euthanasia at day 3 after infection. The tissues were fixed in 10% neutral buffer formalin, processed and embedded in paraffin, sectioned at 5 µm, stained with haematoxylin and eosin and examined by light microscopy in a blinded fashion.

Stability testing in eggs

To test the stability of the vaccine virus on propagation, we made 16 consecutive passages of the virus in embryonated chicken eggs. A 10^{-4} dilution of the virus was made in PBS, and 0·1 mL of the solution was injected into the allantoic cavities of all of four 10-day-old embryonated chicken eggs. Eggs were incubated at 35°C for 1·5–2 days. After incubation, each egg was candled to determine embryo viability before chilling at 4°C. We harvested 2 mL of allantoic fluid from each egg harvested, and samples were pooled together, tested for haemagglutination activity, and then reinjected into another four eggs.

Role of the funding source

The sponsor had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report or decision to submit this manuscript for publication.

Results

Alteration of haemagglutinin cleavage site and virus rescue

The first challenge we faced in producing a vaccine against A/Hong Kong/213/03 (H5N1) was to attenuate the virus in preparation for mass production. Previous experiences have shown that removal of the basic aminoacids at the haemagglutinin cleavage site substantially attenuates pathogenic influenza viruses.^{15–17} Using a PCR-based mutagenesis approach, we replaced the cleavage site encoded by the haemagglutinin gene of A/Hong Kong/213/03 (H5N1) with that of the avirulent A/teal/Hong Kong/W312/97 (H6N1) (figure 1); this modified haemagglutinin gene and the neuraminidase gene of A/Hong Kong/213/03 (H5N1) were cloned individually into the vector pHW2000.¹⁸ The two resulting plasmids and the six plasmids encoding the remaining proteins of PR8¹⁹ were transfected into WHO-approved Vero cells under GMP conditions to rescue the vaccine seed virus, Δ213/PR8. 36–48 h after transfection, isolated areas of cytopathic effect could be seen on the Vero monolayers. Although addition of further 1 µg aliquots of TPCK-treated trypsin every 24 h led to a proportional increase in the cytopathic effect, it was not required for successful virus rescue. The candidate vaccine strain grew to high titres on subsequent amplification in eggs (haemagglutination titres of 1024–2048) and did not cause embryo death. The vaccine seed virus was unable to form plaques on Madin-Darby

A/teal/HK/W312/97 (H6N1)

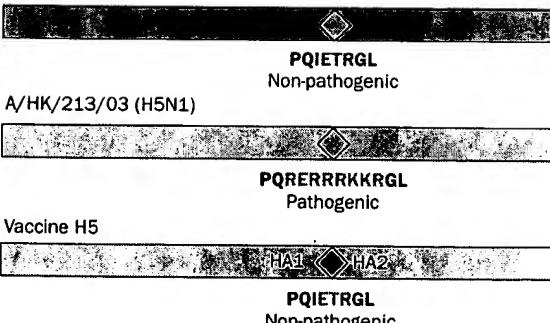


Figure 1: Creation of haemagglutinin protein of candidate vaccine seed

Haemagglutinin protein of the candidate vaccine seed (Δ213/PR8) was produced by replacing the connecting peptide of the A/Hong Kong/213/03 haemagglutinin gene with that of the A/Teal/Hong Kong/W312/97 gene.

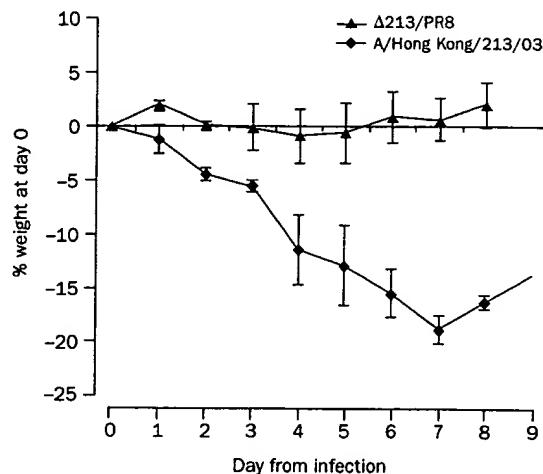


Figure 2: Weight changes of ferrets infected with wildtype A/Hong Kong/213/03 or Δ213/PR8
Vertical bars show SD.

canine kidney (MDCK) cells in the absence of trypsin, a trait consistent with that of influenza viruses that lack the polybasic cleavage site, and was antigenically indistinguishable from the parental H5N1 virus in haemagglutination inhibition assays. The rescued virus was fully sequenced and was identical to the plasmids used in its creation.

Pathogenicity testing of the candidate reference virus

To assess the pathogenicity of the H5N1 vaccine seed virus, we compared the properties of this virus with those of the wildtype A/Hong Kong/213/03 (H5N1) in ferrets and in chickens. By stark contrast with the wildtype virus, which was lethal to all chickens within 48 h of infection, intravenous administration of a 1/10 dilution of Δ213/PR8 did not result in any signs of infection in chickens, and we were unable to detect any virus in swabs of cloacae or tracheae from inoculated birds. Compared with A/Hong Kong/213/03 (H5N1), Δ213/PR8 was attenuated in ferrets that had been inoculated intranasally with 10^6 EID₅₀ of virus. Ferrets infected with A/Hong Kong/213/03 had inappetence and weight loss (figure 2), with one infected animal dying 6 days after infection and a second killed 10 days after infection because of hind-limb paralysis. Infection in these animals was characterised by viral shedding until 7 days after infection and replication of virus in the lower respiratory tract and olfactory bulb (as determined by virus isolation). In the A/Hong Kong/213/03 infected animals, there was a mild mononuclear cell infiltrate in the meninges and tracheal submucosal mucous glands and an extensive bronchopneumonia. The pneumatic infiltrate progressed in severity from the bronchi to the pleura. The bronchi and bronchioles contained sloughed necrotic epithelial cells, numerous mononuclear cells, and a few neutrophils. The alveoli were consolidated with inflammatory cells and fibrin (figure 3). By contrast, those ferrets infected with Δ213/PR8 did not lose weight (figure 2) and seemed to remain healthy during the study (14 days) (figure 3). Virus was detected in the nasal washes of these animals at 5 days but not 7 days after infection, and virus was recovered from the upper respiratory tract only. By light microscopy, the meninges and trachea of the Δ213/PR8 infected ferrets did not have an inflammatory infiltrate and only a few neutrophils were noted occasionally in pulmonary bronchi. Our results clearly show that

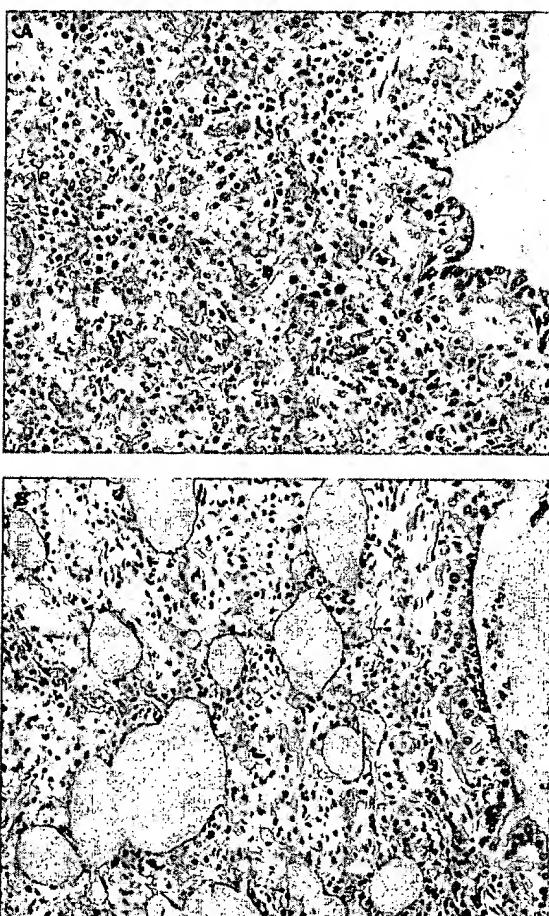


Figure 3: Ferret lung 3 days after infection with wildtype virus (A) and the reverse genetic virus Δ213/PR8 (B)

(A) Alveoli are filled with inflammatory cells and the bronchiolar submucosa is oedematous. (B) Alveoli are free of inflammatory cells and there are a few neutrophils on the surface of the bronchiolar epithelium. Magnification $\times 20$.

Δ213/PR8 was attenuated. In view of our findings, this virus can be safely handled with standard precautions in BSL2 containment facilities.

Stability of non-pathogenic phenotype

Because the mechanisms and requirements for the accumulation of basic aminoacids at the haemagglutinin cleavage site are not entirely understood, we wanted to confirm that the altered cleavage site remained stable on multiple passages in embryonated chicken eggs. Such passaging in eggs would occur in transition and amplification of the reference virus to vaccine stock. The rescued virus was stable on continued serial passage in embryonated eggs, and we did not detect any change in nucleotide sequence of the haemagglutinin cleavage site after 16 passages. There was no evidence of changing pathogenicity of the virus and we noted only one dead embryo at passage 15. No haemagglutination activity was evident in this egg and no embryo death was seen in passage 16, which strongly suggests that the death was not related to virus replication. Haemagglutination titres at each passage ranged from 512 to 2048 with no apparent trend of increasing or decreasing titres in subsequent passages.

Discussion

The rapid response in terms of potential vaccine reference virus production to the 2003 H5N1 outbreak differs strikingly from the response to the 1997 episode. This difference is attributable to the new scientific technology available in 2003 and, just as importantly, to the infrastructure for virus surveillance in Hong Kong developed since 1997. The first case of H5N1 influenza in Hong Kong was in May, 1997; yet several months elapsed before this virus was finally characterised as an H5N1 virus. In 2003, the causative agent was identified only hours after admission of the patients to the hospital. The increased awareness, surveillance, and availability of reagents to identify influenza viruses of all subtypes bode well for the rapid identification of viruses that arise from future interspecies transfer events and for the coordination of international vaccine development by WHO. The timely distribution of candidate viruses is a very important step in the development of vaccines for pandemic emergencies. Despite the heightened security and documentation requirements for shipping and receiving potential bioterrorism agents, the H5N1 and SARS outbreaks have shown that in true emergencies, global distribution is feasible.

Although it is pertinent to prepare for future pandemics by stockpiling potential vaccine strains, the H5N1 situation in 2003—and the ongoing H5N1 outbreaks throughout Asia in 2004 (<http://www.who.int>)—have highlighted the fact that some of the focus of pandemic planning must go into the implementation of technology to rapidly produce vaccines from field isolates. Although viruses similar to A/Hong Kong/213/03 (H5N1) had been circulating in bird populations, these viruses were antigenically distinct, despite high genetic similarities (Guan Y and Peiris JS, unpublished data). That the aminoacid differences are on the globular head of haemagglutinin and seem to be responsible for much of the antigenic difference means that even a vaccine previously prepared from genetically similar precursor viruses might not provide adequate protection. We may well be faced with potential pandemic situations in the future and the rapid production of a matched vaccine will be needed—a point again highlighted by H5N1 outbreaks in 2004. Although the reference virus described in this report was prepared from a virus isolated in a similar geographic region and only a year earlier, it shares only limited antigenic cross-reactivity to the 2004 H5N1 viruses. Hyperimmune sheep serum samples produced against the purified haemagglutinin of Δ213/PR8 has at least a six-fold reduced haemagglutination inhibitory activity against A/Vietnam/1203/04 as compared with A/Hong Kong/213/03. As our findings show, we have the technical capabilities to respond rapidly to outbreaks with a safe and stable reference virus, but there is still much to be accomplished before such viruses can be fully used in pandemic and interpandemic influenza vaccine production.

The use of reverse genetics introduces a number of new processes into influenza vaccine manufacture that are not encountered with standard reassortment methods. One of the most obvious is the need for cultured cells. Although both Vero¹⁸ and MDCK^{19,20} cells are in development as substrates for the growth of influenza vaccine, there are additional requirements for the use of cells in reverse genetics. Unfortunately, the number of suitable cell lines is very small. In addition to the regulatory requirements, the choice of cell is also limited by the technology. The plasmid based reverse-genetics systems¹⁰⁻¹² use the species-specific human RNA polymerase I promoter, which

necessitates the use of cells from primate origin. The Vero cell line is probably the only option currently able to meet both regulatory and technical demands. We have shown that Vero cells can be used to successfully rescue H1N1, H3N2, H6N1, and H9N2 viruses on the PR8 backbone using the 8-plasmid system.²¹ Others have demonstrated the suitability of Vero cells for alternative influenza virus reverse-genetics systems.¹⁰ Although cultures of Vero cells are easily obtained, only cells from fully tested and licensed cell banks are likely to be acceptable for vaccine manufacture. This issue must be acknowledged and access to such cells must be incorporated as part of future pandemic plans.

That future threats of influenza pandemics will be addressed by the use of the technology described in this report seems inevitable. Despite the presence of low pathogenic surrogate strains, the recent human death from influenza-like illness caused by highly pathogenic H7N7 virus in the Netherlands²² reinforces the fact that future outbreaks will probably occur in which this reverse-genetics technology provides the logical—and, possibly, the only—way to respond rapidly and effectively. Although our response to the outbreak of H5N1 influenza in 2003 has shown that current scientific capabilities are sufficient to respond to the threat, there are still legal and infrastructural barriers to be overcome.²³ These barriers include licensing and intellectual property issues surrounding what is, essentially, a genetically modified organism. Yet, these difficulties are not insurmountable and pandemic scares such as the 2003 and ongoing 2004 H5N1 outbreaks are forcing commercial and regulatory parties to address these issues with some urgency. With the development of the 2003 H5N1 vaccine reference virus, and ongoing attempts to create the same for the 2004 virus, the challenge in responding to a threat of an influenza pandemic must now be supported by the large-scale manufacture of the vaccine and by clinical trials of a new vaccine manipulated by reverse genetics.

Contributors

R J Webby, D R Perez, J S Coleman, J H Knight, E I Tuomanen, R G Webster designed the study; R J Webby did much of the construction of the vaccine seed virus; D R Perez developed and constructed plasmid templates; Y Guan and J S Peiris characterised and isolated the initial H5N1 virus; J E Rehg participated in the design and analysis of animal safety testing of the candidate H5N1 vaccine seed virus; E A Govorkova participated in the safety testing of the candidate H5N1 vaccine seed virus; L R McClain-Moss participated in the preparation of GMP documentation of the process and was involved in the reconstitution of the vaccine seed virus.

Conflict of interest statement

None declared. The corresponding author has had full access to all the data in the study and has had the final responsibility for the decision to submit this manuscript for publication.

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